

1999

Production of L-(+) Lactic Acid From Blackstrap Molasses by Lactobacillus Casei Subspecies Rhamnosus ATCC 11443.

Narumol Thongwai

Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation

Thongwai, Narumol, "Production of L-(+) Lactic Acid From Blackstrap Molasses by Lactobacillus Casei Subspecies Rhamnosus ATCC 11443." (1999). *LSU Historical Dissertations and Theses*. 7019.
https://digitalcommons.lsu.edu/gradschool_disstheses/7019

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI[®]

**Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600**

**PRODUCTION OF L- (+) LACTIC ACID FROM BLACKSTRAP MOLASSES BY
Lactobacillus casei SUBSPECIES *rhamnosus* ATCC 11443**

A Dissertation

**Submitted to The Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirement for the degree of
Doctor of Philosophy**

in

The Department of Biological Sciences

by

**Narumol Thongwai
B.S., ChiangMai University, 1988
August 1999**

UMI Number: 9945746

UMI Microform 9945746
Copyright 1999, by UMI Company. All rights reserved.

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

DEDICATION

**To my father for forging the desire in me to step higher in my education and career regardless
his passing away in the year I entered the program.**

To my mother for passing her strength to me.

To my father and mother for their love, support, patience and encouragement.

ACKNOWLEDGMENTS

I deeply thank my advisor, Dr. Donal Forest Day, for his fruitful discussions throughout this work. From the beginning, he has been my mentor not only in sciences but also in many other topics. His advise, support, understanding and encouragement carried me through the difficult times.

My sincere appreciation goes to Dr. John Battista, Dr. Alan Biel and Dr. Greg Jarosik for spending their valuable time to discuss and review my work. Their advice and constructive criticism made this work possible.

Dr. Roger Laine gave me not only his intellectual insight into this work but also he opened my eyes to see other facets of sciences. I do not know how to thank him enough.

Ms. Cindy Henk always gave me some solutions of my problems in her field. Her beautiful work on the scanning electron microscopy are shown in my dissertation.

I would like to express my deeply appreciation to Ms. Durriya Sarkar at the Audubon Sugar Institute (ASI). She helped and gave me advice through my work and many other topics. Her kindness will never be forgotten. My great appreciation goes to Dr. Lorraine Day for her kindness at artworks and her valuable point of views in my project.

Mr. Mickey Brou at the ASI was the person whom I could not forget. He always helped me whenever I called for him. His major contribution was in the pilot plant. Ms. Mei Wu (former employee of the ASI) helped me a great deal on the HPLC for sugar analysis.

I would like to thank all the graduate students in both the Department of Microbiology and the Audubon Sugar Institute. Some of them had given me good times and encouraged me to move one step further toward my degree while the others made me stronger by giving me hard times to deal with. Foremost of these was Dr. C. Mark Ott who introduced microbiology and basic engineering to me. Since I began the program, he has helped me in so many ways that I cannot mention them all. His advice, support, patience and friendship will never be forgotten.

I would like to thank all my Thai friends for their cherished motivations. Dr. Parin Chaivisuthangkura has provided me a wonderful friendship and encouraged me to step further toward my degree every time I was depressed and wanted to give up the program. Ms. Watchanan Wongsena supported me in so many ways. Her unusual ideas always made me smile. Mr. Kasidit Chanchio, Mr. Pongtarin Lotrakul, Ms. Orachos Napasintuwong and Dr. Witoon Prinyawiwatkul provided me good times during these years in LSU. I will be grateful for their friendship forever.

I would like to thank the Royal Thai government for providing me education funds. Without this sponsorship, I would have never been here today.

Finally, I would like to thank all the members of my family. My parents, Juntree and Bunpan Thongwai, were always supportive and forging me to step higher in my education and career. My eldest brother, Lieutenant Colonel Chusak Thongwai provided my education fund and always supported me in any endeavor I attempted. My older brother Mr. Sunan, my older sister Ms. Supawadee, my little brother Puwadol and my eldest sister-in-law Mrs. Watanaporn Thongwai were always trying to understand and accept me for who I am. I would like to thank all my nieces (Sakuntara, Surassa, Siwaporn and Sasitorn Thongwai) and nephews (Apiratana and Suthisak Thongwai) for bringing fun and joy into my life. Without any of these contributors, I would never be what I am.

TABLE OF CONTENTS

| | |
|---|------|
| DEDICATION..... | ii |
| ACKNOWLEDGEMENTS..... | iii |
| ABSTRACT..... | viii |
| INTRODUCTION..... | 1 |
| LITERATURE REVIEW..... | 4 |
| I. Lactic Acid..... | 4 |
| A. Physical and chemical properties..... | 4 |
| B. Natural occurrence..... | 4 |
| II. Lactic Acid Applications..... | 6 |
| A. Food and food-related industries..... | 6 |
| B. Non-food uses..... | 7 |
| C. Lactic polymers..... | 7 |
| III. Lactic Acid Syntheses..... | 13 |
| A. Chemical synthesis..... | 13 |
| B. Biosynthesis of lactic acid..... | 15 |
| B.1 Organisms..... | 15 |
| B.2 Carbohydrate metabolism of the lactobacilli..... | 22 |
| B.3 Lactic acid configuration and lactate dehydrogenase..... | 27 |
| B.4 Solute transport..... | 27 |
| B.5 Regulation of lactic acid synthesis..... | 33 |
| IV. Industrial Processes for Lactic Acid Fermentation..... | 36 |
| A. Batch fermentation..... | 36 |
| B. Substrate sources..... | 36 |
| V. Product Recovery..... | 37 |
| VI. Alternate Technologies for Lactic Acid Fermentation and Purification..... | 38 |
| VII. Cell Immobilization..... | 40 |
| A. Covalent coupling and cross-linking..... | 40 |
| B. Affinity (biospecific) immobilization..... | 40 |
| C. Adsorption..... | 41 |
| D. Entrapment..... | 41 |
| VIII. Molasses..... | 41 |
| MATERIALS AND METHODS..... | 47 |
| I. Organism and Maintenance..... | 47 |
| II. Analytical Methods..... | 47 |
| A. Cell density determination..... | 47 |
| B. Cell mass determination..... | 47 |
| C. Determination of sugars..... | 48 |
| C.1 Reducing sugars..... | 48 |
| C.2 HPLC sugar analysis..... | 48 |
| D. Determination of L- (+) lactic acid..... | 49 |

| | |
|---|-----|
| III. Growth..... | 49 |
| A. Shake flasks..... | 49 |
| B. 1 liter fermentors..... | 49 |
| IV. Cell Immobilization and Bioreactors..... | 50 |
| A. Solid supports..... | 50 |
| B. Bench scale continuous bioreactor..... | 50 |
| B.1 Bench (10 ml) bioreactor..... | 50 |
| B.2 Chemostat (400 ml) bioreactor..... | 50 |
| C. Pilot plant..... | 50 |
| V. Optimization of Fermentations..... | 53 |
| A. Temperature..... | 53 |
| B. Initial pH..... | 53 |
| C. Sodium azide..... | 55 |
| D. Flow rates..... | 55 |
| E. Extracellular lactic acid..... | 56 |
| F. Effect of substrate concentration on lactic acid production..... | 56 |
| G. Effect of nutrient supplemented molasses on lactic acid production and cell growth..... | 57 |
| H. Effect of molasses inversion on lactic acid fermentation..... | 57 |
| I. Effect of molasses clarification on lactic acid production..... | 58 |
| J. Effect of solid supports on cell immobilization and lactic acid production..... | 58 |
| VI. Adsorption of Lactic Acid by Activated Carbon..... | 58 |
| VII. Purification of Lactic Acid..... | 59 |
| VIII. Scanning Electron Microscopy (SEM)..... | 59 |
| IX. Calculated Parameters..... | 59 |
| RESULTS..... | 81 |
| I. Lactic Acid Production..... | 61 |
| A. Shake flask studies..... | 61 |
| A.1 Growth and production on Lactobacillus MRS media..... | 61 |
| A.2 Growth and production on blackstrap molasses..... | 70 |
| B. Fermentor studies (pH controlled)..... | 83 |
| B.1 Lactic acid production on Lactobacillus MRS media..... | 83 |
| B.2 L- (+) lactic acid production on molasses..... | 83 |
| C. Immobilized cell fermentation..... | 95 |
| C.1 Solid supports for cell immobilization:electron microscopy..... | 95 |
| C.2 Adsorption of lactic acid by activated carbon..... | 95 |
| C.3 Immobilized cell bioreactors.(10 ml)..... | 95 |
| C.4 Immobilized cell chemostat (400 ml)..... | 115 |
| D. L - (+) lactic acid production (pilot plant)..... | 115 |
| D.1 Without pH control..... | 115 |
| D.2 With pH control..... | 120 |
| II. Purification of Lactic Acid..... | 120 |
| III. Financial Analysis of Lactic Acid Production..... | 125 |
| A. Batch fermentation..... | 125 |
| B. Continuous, immobilized fermentation..... | 125 |
| DISCUSSION..... | 128 |

| | |
|-----------------------|-----|
| LITERATURE CITED..... | 138 |
| VITA..... | 148 |

ABSTRACT

Lactobacillus casei subspecies *rhamnosus* grew on non-supplemented blackstrap molasses. The bacteria grew best at the optimal temperature of $40 \pm 2^{\circ}\text{C}$, and at pH 5.5. Addition of yeast extract to molasses shortened the lag phase and increased cell mass. In batch fermentations without pH control, the highest ratio of lactic acid produced to cell mass was 12 from non-supplemented molasses. For pH controlled batch fermentations, the ratio's of lactic acid produced/cell mass were 81, 79, 51 and 31 for 0, 0.2, 0.4 and 0.8% yeast extract supplementation, respectively. Non-supplemented molasses, 8% Brix, produced 28 ± 2 g/l of lactic acid in batch fermentations, pH 5.5. Molasses clarification reduced lactic acid production by 53%. Inversion of the sucrose in molasses with 0.2% invertase resulted in lactic acid production of a molar conversion of 1.99, with no residual sugars. Invertase also acted as a better nutrient supplement than did yeast extract. *L. casei* subsp. *rhamnosus* was inhibited by L- (+) lactic acid at a K_i of 120 mM when grown on MRS media, and 360 mM on molasses. The bacteria were inhibited by sodium azide at a K_i of 2.1 mM.

L. casei subsp. *rhamnosus* immobilized onto solid supports. At a dilution rate of 0.2/hr, the most lactic acid (14 ± 1 g/l) was produced from non-supplemented molasses. Doubling the dilution rate halved the yield of lactic acid. Residual fermentable sugar in molasses was primarily sucrose. At dilution rates of 0.2 and 0.4/hr, nutrient supplementation of molasses did not enhance the production. A 3-stage mini-pilot plant, 5.4 liters working volume, was constructed. Most of the lactic acid (78%) was produced in the first segment of the bioreactor. Cost of carbohydrate and nutrient supplement of lactic acid production from batch fermentation was \$0.35/kg, and from the immobilized bioreactors was \$0.24/kg. If molasses is resold, the production cost of lactic acid from immobilized bioreactors drops to \$0.075/kg.

INTRODUCTION

Lactic acid was discovered in 1780 by the Swedish chemist Scheele in sour milk (Datta, *et al.*, 1995 and Ruter, 1975). It was the first organic acid to be produced commercially, by fermentation, with production beginning in 1881 (Ruter, 1975, Severson, 1998 and Lockwood, 1979). By 1894, the production of this organic acid was 10,000 pounds per year. By 1917, production was to a million pounds per year (Inskeep, *et al.*, 1952). It is currently 120 to 130 million pounds (Datta, *et al.*, 1995 and Severson, 1998) of L- (+) lactic acid with a value of about 150 million dollars (McCoy, 1998). Worldwide demand for lactic acid is growing at a rate of approximately 12 to 15% a year. This acid has been called a "commodity chemical sleeping giant" (Lipinsky, *et al.*, 1986 and Bahner, 1994).

In the USA until 1963, lactic acid was produced solely by fermentation, when Sterling Chemicals, Inc., started producing lactic acid by a chemical process using petroleum by-products, supplying nearly half the American demand for lactic acid. In 1996, Sterling abandoned the lactic acid business, leaving lactic acid production again exclusively to fermentation companies (Severson, 1998 and McCoy, 1998). In the early 1990s, Ecological Chemical Products (EcoChem), a joint venture of E.I. du Pont Nemours & Co., and Con Agra, Inc., (in a projected 20 million pound-per-year plant) produced only 1 to 2 million pounds of lactic acid by fermentation of whey permeate. This failure led to the termination of the project (Lerner, 1995). In 1993, the current leader in basic chemical fermentation, Archer Daniels Midland (ADM), entered the lactic acid business and produced, in a facility designed for 40 million pounds-per-year, 10 million pounds of lactic acid from corn sugar. It is expected they will expand production in the future (McCoy, 1998 and Lepree, 1995). In mid-1996, an A.E. Staley and Cargill-Purac joint venture announced they would enter the lactic acid fermentation business (McCoy, 1998 and Lunt, 1998). In December 1997, Cargill created a 50-50 joint venture with Dow Chemical, a polymer producer, to produce polylactic acid (PLA) (Thayer, 1997). Worldwide significant suppliers of lactic acid are CCA Biochem b.v., from the

Netherlands, and its subsidiaries in Brazil and Spain (fermentation process) and a smaller manufacturer Musashino, in Japan (chemical technology) (Datta, *et al.*, 1995 and Kharas, *et al.*, 1994). With a potential market for lactic acid in polymer production, the demand for lactic acid may reach as high as 400 to 500 million pounds per year (Severson, 1998 and Kharas, *et al.*, 1994). For lactic acid to become attractive for use in biodegradable thermoplastics, production costs must be reduced.

Lactic acid is usually marketed as either the D or L isomer, which means specific strains of microorganisms are used for the production. The production costs can be broken down into two stages, the fermentation costs and the product recovery costs. The carbohydrate sources presently used for lactic acid production are molasses (beet or cane), whey or whey permeates and starch hydrolysates. Although these substrates are low cost, they need to be supplemented with a source of vitamins, amino acids and mineral salts. The nutrient supplements commonly used in lactic acid production are yeast extract or corn steep liquor. The nutrient supplement is normally more expensive than the carbohydrate source. Lactic acid is also characterized by end-product inhibition, which influences both amount of lactic acid formed and costs for downstream process. The current fermentation technology shows fluctuations in yield due to batch-to-batch variation, low productivity and high concentration wastes (salts and BOD). These increase the cost of lactic acid production.

This project is a study on the feasibility of reducing the cost of production of L- (+) lactic acid by using blackstrap molasses as a feed stock in conjunction with continuous fermentation. The bacteria was immobilized on solid supports in order to increase numbers of cells in the fermentation, increasing productivity and lowering production cost. *Lactobacillus casei* subspecies *rhamnosus* was the organism of choice as it produces only L- (+) lactic acid from molasses. Fermentation conditions, both batch and immobilized, were experimented. Also, nutrient supplementation to molasses and other methods of enhancing production were studied. A pilot plant was designed, based on laboratory results, and tested for production efficiency. Economic analysis of substrate utilized for L- (+) lactic acid production selected

approaches for minimizing production costs, with the ultimate goal of opening a new market for Louisiana sugar cane molasses.

LITERATURE REVIEW

I. Lactic Acid

A. Physical and chemical properties. Lactic acid, or 2-hydroxypropionic acid, ($C_3H_5O_3$), is a common organic acid. The name lactic acid is derived from the Latin word for milk (lac) because when raw milk sours naturally, the lactose present is largely converted into lactate. Lactic acid is nonvolatile, odorless, colorless and has a mild acidic taste, in contrast to other food acids. It is mildly corrosive. Lactic acid is classified as GRAS (Generally Recognized As Safe) for use as a general purpose food additive by the FDA (Food and Drug Administration) in the U.S. and by other regulatory agencies worldwide (Datta, *et al.*, 1995 and Garvie, 1980).

Lactic acid occurs in 2 isomeric forms, D- or L- (Fig.1). The content of carbon, hydrogen and oxygen are 40, 6.71 and 53.29%, respectively.

a. Racemic or DL- lactic acid has a melting point of $16.8^{\circ}C$ and a boiling point of $122^{\circ}C$. The dissociation constant of DL- lactic acid at $25^{\circ}C$ is 1.38×10^{-4} . Its density is 1.2 g/cm^3 (Ruter, 1975).

b. D- (-) lactic acid, (R)-2-hydroxypropanoic acid or levorotatory lactic acid, has a pK of 3.83. It dissolves readily in water, alcohol, acetone, ether and glycerol.

c. L- (+) lactic acid, dextrorotatory lactic acid, sarcosylactic acid or paralactic acid, has a pK of 3.79. It is the only physiologically active form (Buchta, 1983 and Nagodawithana, 1998). L- (+) lactic acid dissolves more readily in water than the racemic acid (DL form).

Both D- (-) and L- (+) lactic acid are insoluble in chloroform. D- (-) lactic acid salts are dextrorotatory while L- (+) lactic acid salts are levorotatory (Merck index, 1989).

B. Natural occurrence. Racemic lactic acid is produced as a result of bacterial fermentation in sour milk; molasses; apples and other fruits; tomato juice, beer, wine, opium, ergot, foxglove; in fermented foods such as sauerkraut, yogurt, buttermilk, sourdough breads; and in higher plants during germination (Datta, *et al.*, 1995 and Merck index, 1989).

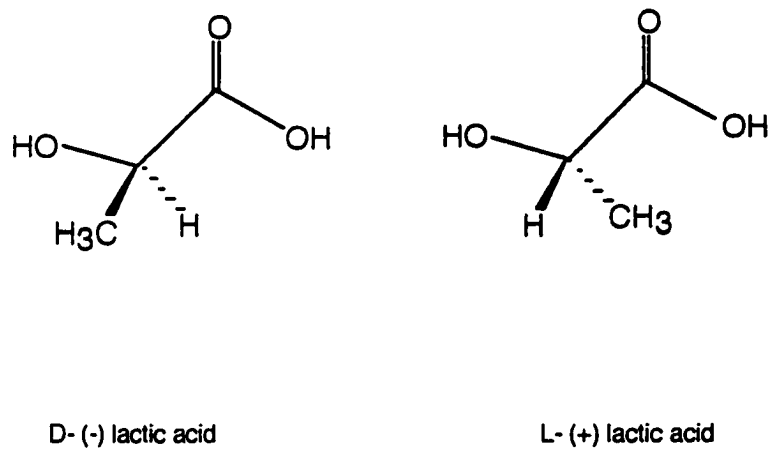


Figure 1. Configurations of L- (+) and D- (-) lactic acid

D- (-) lactic acid is not a human metabolite. When ingested, it produces illness in infants younger than 6 months of age (Benninga, 1990). D- (-) lactate is oxidized or excreted in the urine. D- (-) lactic acid can be produced from racemic lactic acid. L- (+) lactic acid is a metabolic intermediate of human and animals. It is present in the blood, muscles, liver, kidney, thymus gland, human amniotic fluid, etc. It is metabolized back to glycogen, in humans, if it is not utilized.

II. Lactic Acid Applications

A. Food and food-related industries. Approximately 85% of lactic acid produced is used in food and food-related industries (Datta, *et al.*, 1995). Lactic acid is an acidulant, buffer, chelating agent, preservative, flavour enhancer and texture modifier. Food grade lactic acid has 50-65% acidity (Atkinson and Mavituna, 1983). It is a good preservative and is used as a pickling agent for sauerkraut, olives and pickled vegetables. Lactic acid is added to inhibit bacterial spoilage and as an acidulant in processed food such as; candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise and processed eggs. Very often, it is used in conjunction with other acidulants (Datta, *et al.*, 1997, Duxbury, 1993 and Purac, 1989). In soy sauce production, lactic acid provides the appropriate conditions for yeast growth and ethanol production, influencing soy sauce flavor and taste (Iwasaki, *et al.*, 1992 and Iwasaki, 1993).

Recently, lactic acid and its salts have been used in the disinfection and packaging of carcasses, particularly poultry and fish. Lactic acid reduces growth of the anaerobic spoilage microorganisms, such as *Clostridium botulinum*, and increases shelflife (Datta, *et al.*, 1995, Anders, *et al.*, 1989 and 1991).

More than 50% of the lactic acid used in food-related applications is in the form of an emulsifying agent, an ester of lactate salts and a long chain fatty acid. Calcium or sodium, stearoyl-2-lactylate, glyceryl lactostearate, and glyceryl lactopalmitate are the most common lactate esters. The calcium salt is a good dough conditioner while the sodium salt is both a conditioner and an emulsifier in yeast leavened bakery products. In prepared cake mixes,

glyceryl lactopalmitate improves cake texture while glyceryl lactostearate is used to increase cake volume to meet mixing tolerances. Production of these emulsifiers, particularly the stearoyl-2-lactylates requires high purity, heat-stable lactic acid (Datta, *et al.*, 1995 and Purac, 1989).

B. Non-food uses. Lactic acid is used as acidulant for deliming hides, and as a pluming agent in leather tanning industries (Ruter, 1975). Technical grade lactic acid has been used in various textile finishing operations and in acid dyeing of wool. Although cheaper inorganic acids are now used in these applications, this market may reopen to lactic acid because of increasing restrictions on disposal of waste salts.

Currently, lactic acid is used in applications where the functional specialty of the molecule is desired. It is used for adjusting the pH of hardening baths for production of cellophane that is used in food packaging. Lactic acid is also used as a terminating agent for phenol-formaldehyde resins, an alkyd resin modifier, a solder flux, a lithographic and textile printing developer, in adhesive formulations, electroplating and electropolishing baths and as a detergent builder (with maleic anhydride to form carboxymethylsuccinic acid compounds). Only 5 to 10% of total lactic acid production is used in these applications (Datta, *et al.*, 1995, Purac, 1989 and Van Ness, 1981).

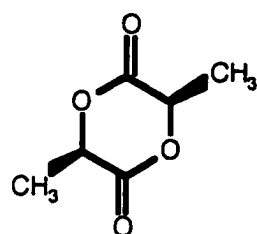
Lactic acid and its esters are used in topical ointments, lotions, parental solutions and dialysis applications (Van Ness, 1981, Holland, 1986 and Dunn, 1988). Dialysis and parental solution applications contain the sodium salts of lactic acid. The calcium salt is used in calcium-deficiency therapy and as an anti-caries agent. Lactic acid and its esters are widely used as humectants in cosmetic applications, as they are more effective than other natural products and polyols (Van Ness, 1981 and Purac, 1989). Ethyl lactate is the active ingredient in anti-acne preparations. These above applications require both heat-stable and high purity lactic acid.

C. Lactic polymers. In 1932, Carothers first produced an aliphatic polyester of low molecular weight from lactic acid, but it had poor mechanical properties (Holten, 1971). In

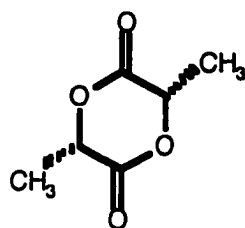
1954, Dupont patented the production of a high molecular weight polylactic acid. However, the development was terminated because of the hydrolytic degradability of the polymer (Lowe, 1954). In 1972, Ethicon produced high-strength co-polymers of lactic and glycolic acids. These polymers are now used as biocompatible fibers in resorbable sutures. They are slowly hydrolyzed within the body to the constituent acids. For many years, growth in polylactic acid production has been inhibited by the high cost of the lactic acid monomer. In late 1980s, new materials (see section VI) for lactic acid production by fermentation introduced lower-cost lactic acid than the petrochemically-derived product. Cargill, Inc. Minneapolis, MN now operates the world's largest polylactic acid facility (Lunt, 1998).

Polylactic acid (PLA) production requires high purity monomers and a heat-stable, optically active lactic acid, in the L- isomeric form, and/or its ester. There are two routes now used to produce lactic acid polymer. The first method is the solvent-free and distillation processes used by Cargill to produce a range of polymers (Gruber, *et al.*, 1992). Advantage of this process lies in the ability to go from a low-molecular-weight polylactic acid, with controlled depolymerization, to the cyclic dimer, lactide, (Fig. 2). The lactide is maintained in the liquid form and can be purified by distillation, if needed. The controlled depolymerization of the low molecular weight polymer to lactide, with subsequent lactide polymerization forms high molecular weight PLA (Fig. 3) (Lunt, 1998). An alternative method is a solvent-based process where high-molecular-weight polymer is produced by direct condensation, with azeotropic distillation used to continuously remove the water of condensation (Enomoto, *et al.*, 1994).

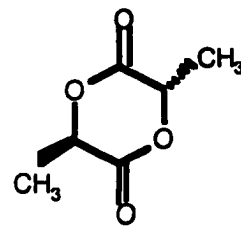
L- (+) lactic acid can racemize to D- (-) lactic acid during PLA production. By controlling residence time and temperature, in combination with catalyst type and concentration, the ratio and sequence of D- and L- lactic acid units in the final polymer can be controlled. The optical isomeric composition of the polymer significantly affects crystallization kinetics and its ultimate crystallinity, which is influential in the determining the polymer melting point (Lunt, 1998 and Kolstad, 1996). D- and L- lactides have a melting point of 97°C while the meso- lactide (optically inactive form) has a melting point of 52°C. Poly (L- lactide), crystalline, has a melting



D- lactide



L- lactide



meso- lactide

Melting point ($^{\circ}\text{C}$) : 97

97

52

Figure 2. The three stereoisomeric forms of lactide.

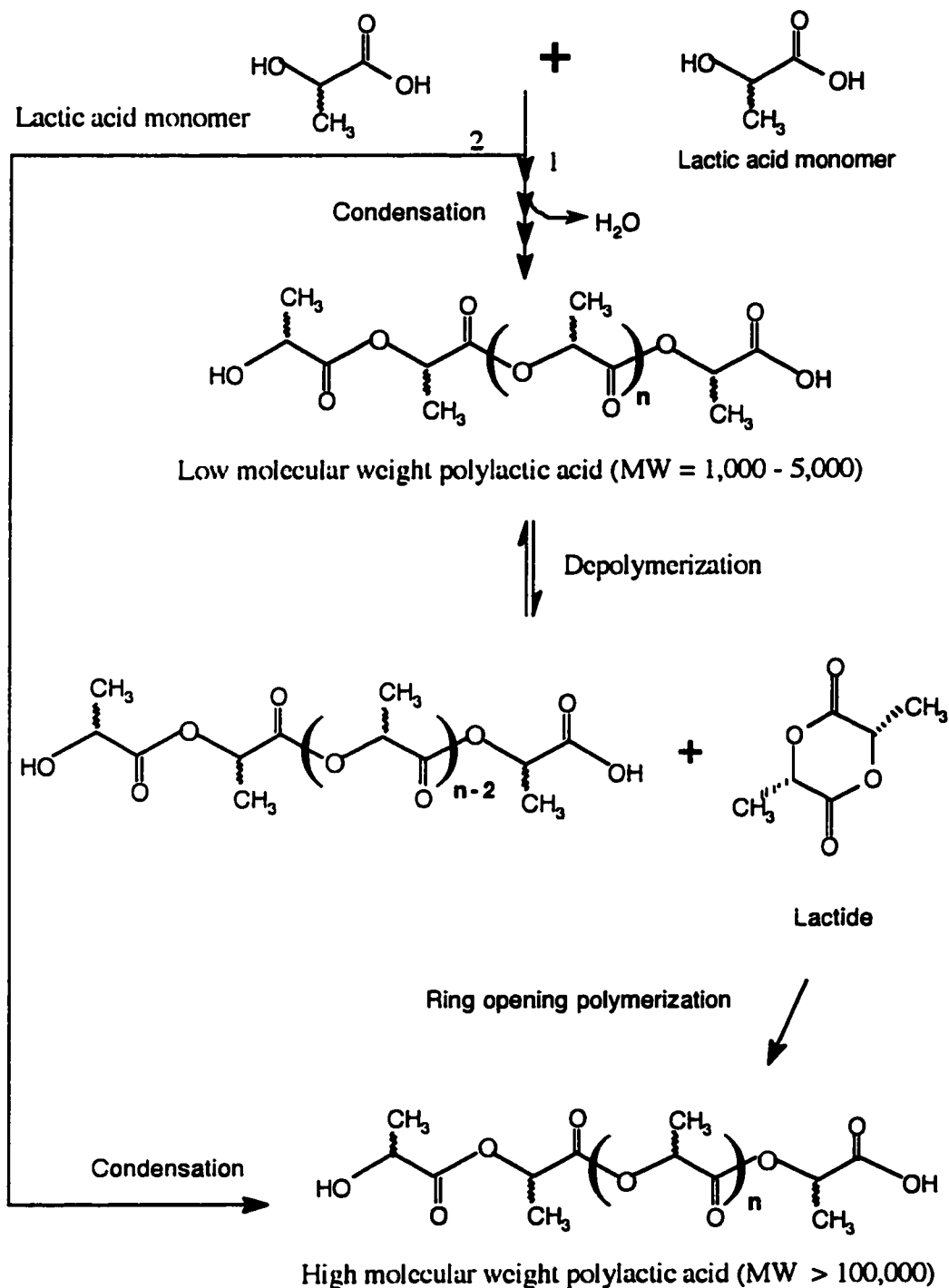


Figure 3. Lactic acid polymerization. Process 1 shows a controlled polymerization, while process 2 shows direct condensation

point of 180°C. Introduction of meso- lactide into the poly L- lactide polymer decreases the melting point to as low as 130°C. PLA polymers can range from amorphous glassy polymers with a glass transition of 60°C to semicrystalline/highly crystalline products with the melting points ranging from 130-180°C. PLA plastics have a greater flexural modulus than polystyrene, they are resistant to fatty foods and dairy products, make good flavour and aroma barriers, have good heat sealability and a high surface energy that allows for easy printability.

Polymers and co-polymers of L- lactide, with other functional monomers such as glycolide, caprolactone and polyether polyols are biocompatible, body absorbable and have a reasonable blood compatibility (Pennings, 1989). In medical applications, PLA polymers have been used in controlled drug release systems, prostheses and surgical sutures. PLA polymer, prepared as microcapsules for use in a controlled drug release system in order to preserve the activity of the drug and to eliminate the side effects of the drug on the other part of the body, was first reported in 1976 (Mason, *et al.*, 1976, Anderson, *et al.*, 1976 and Piskin, *et al.*, 1994). Drugs can be encapsulated in either of two microcapsule morphologies, reservoir or monolithic microencapsulation (Fig. 4) (Tice, *et al.*, 1984, Ogawa, 1992 and Ogawa, 1997). Drugs are encapsulated into PLA polymers by either the solvent evaporation process or the phase separation process (Chang, 1976). Drugs are released more rapidly from poly (L-lactic acid) than from combined poly (DL-lactic acid) and poly (L-lactic acid) microcapsules. Possibly, because poly (L-lactic acid) polymer is crystalline while poly (DL-lactic acid) polymer is amorphous. The membranes formed by poly (DL-lactic acid) are more uniform, reducing drug leakage. Microcapsules made from poly (L-lactic acid) are smaller than those containing poly (DL-lactic acid), giving higher surface area, increasing drug release rates for equivalent volumes of microcapsule (Yu, *et al.*, 1998).

PLA polymers have been used for controlled release of fertilizers and pesticides. The PLA plastics, "green solvents and esters", are now used as single-use environmentally friendly disposable products. A flexible film product suitable for kitchen waste biobags, bin liners and lawn and leaf applications is available in the marketplace. Many new PLA products

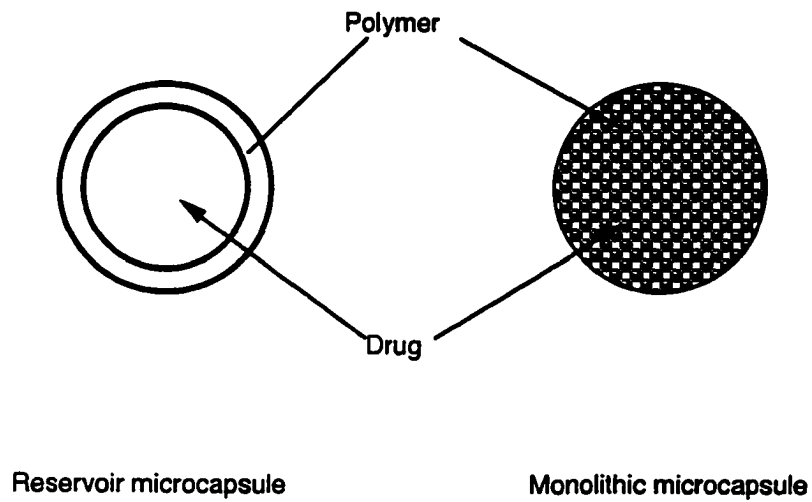


Figure 4. Internal structure of reservoir versus monolithic microcapsules (Ogawa, 1997)

such as thermoformed containers, non-wovens, paper-coated and injection-moulded articles are being developed (Severson, 1998, Lunt, 1998, Datta, *et al.*, 1995 and Kharas, *et al.*, 1994).

PLA polymers are biodegradable aliphatic polyesters. The ester linkages are hydrolyzed by water. This hydrolytic process is controlled by temperature and humidity. At high humidities and at temperatures around 55-70°C, the polymers rapidly degrade. However, at lower temperatures and/or lower humidities, the PLA product is stable. As microorganisms cannot degrade the high molecular weight polymers, PLA polymers are useful for product storage and in applications requiring food contact. When the molecular weight of the polymer decreases to less than 10,000, it can be hydrolyzed by soil microorganisms, ultimately forming carbon dioxide and water (Yu, *et al.*, 1998 and Lunt, 1998). Co-polymers of lactic and glycolic acids (GA) do not biodegrade in mammalian systems (Li, *et al.*, 1990 and Vert, *et al.*, 1991 and 1994). They are abiotically degraded inside the body, and the oligomers are excreted before they can be digested by cells. Thus, polymers of PLA and PGA are said to be bioresorbable in mammalian systems and bioassimilable outside. Polymers of PLA have been reported to be degraded by *Fusarium moniliforme* and *Penicillium roqueforti* (Torres, *et al.*, 1996).

III. Lactic Acid Syntheses

A. Chemical synthesis

Chemical synthesis of lactic acid produces a racemic lactic acid mixture. The process starts by combining of hydrogen cyanide and acetaldehyde, in a liquid phase reaction at atmospheric pressure, producing lactonitrile. The crude lactonitrile is recovered and purified by distillation and then is hydrolyzed into lactic acid using either concentrated sulfuric or hydrochloric acid, producing an ammonium salt as a by-product. This crude preparation is esterified with methanol to produce methyl lactate. Methyl lactate is recovered, purified by distillation, and then hydrolyzed under acidic conditions to produce a purified lactic acid, which is further concentrated and packaged. The methanol is recycled to the process (Fig. 5).

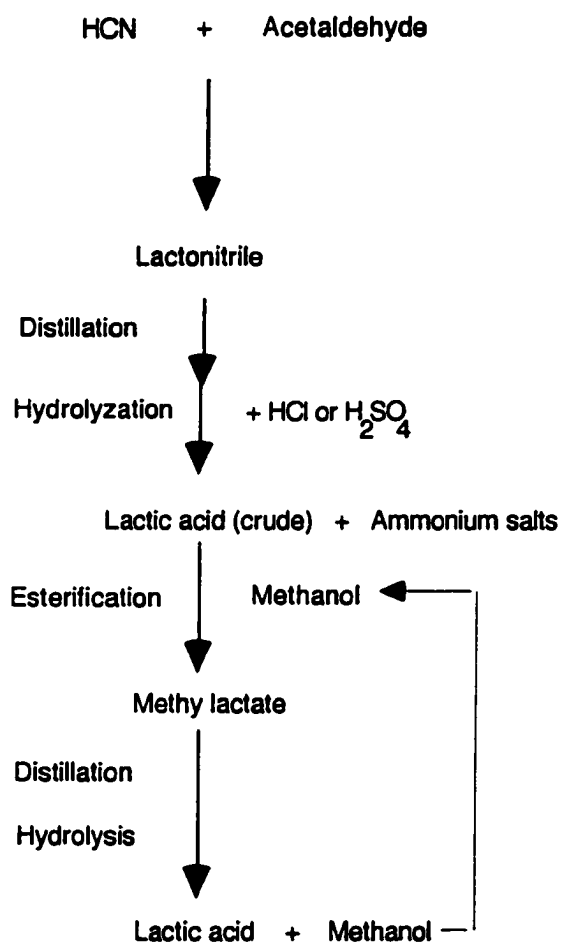


Figure 5. Chemical synthesis of lactic acid

There are other routes for chemically synthesizing of lactic acid, for example: oxidation of propylene glycol; reaction of acetaldehyde with carbon monoxide and water at elevated temperatures and pressure; hydrolysis of chloropropionic acid and nitric acid oxidation of propylene. However, none of these are commercial (Datta, *et al.*, 1995). Because of the growing demand for lactic acid for biodegradable thermoplastics, there is a need for pure chiral forms, D- or L- lactic acid. Chemical synthesis produces a racemic mixture of lactic acid, D and L isomeric forms.

B. Biosynthesis of lactic acid

B.1 Organisms. Many microorganisms produce lactic acid, but most of the commercially used microorganisms are in the genus *Lactobacillus*, which belongs to the family Lactobacteriaceae (Severson, 1998, Axelsson, 1993, Kandler and Weiss, 1986, Vickroy, 1985 and Miall, 1978). The lactobacilli are the members of Lactic Acid Bacteria (LAB) which are a diverse group of Gram-positive, non-spore-forming bacteria, which occur as cocci or rods, and are grouped together by their ability to form lactic acid as a sole or primary end-product from carbohydrate metabolism; either by a homolactic or a heterolactic pathway. The Lactic Acid Bacteria consist of bacteria in the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Vagococcus*, *Aerococcus*, *Alloiococcus*, *Tetragenococcus*, *Atopobium* and *Bifidobacterium* (Schleifer, *et al.*, 1995). Unlike mammalian systems, which produce only L- (+) lactic acid, LAB may produce D, L, or racemic lactic acid (Holzapfel, *et al.*, 1995).

***Lactobacillus* species:** Lactobacilli are Gram-positive, non-spore forming rods or coccobacilli with the rod diameter of 0.5-1.1 microns, and a DNA G+C content between 32-53 mol%. They do not possess catalase, however, a pseudocatalase is found in *L. mali*. They are generally considered non-motile, although peritrichous flagella and motility have been reported for *L. ruminis*, *L. yamanashiensis* and *L. agilis* (McGroarty, 1994). Lactobacilli are strictly fermentative, aerotolerant, acidophilic and fastidious. Their growth temperature ranges from 2-53°C but normally they have an optima around 30-40°C with the optimal pH for growth

between 5.5 and 6.2. *Lactobacillus* growth ceases when the culture pH reaches 3.6-4.0, depending on the species and strain. Generally, they are the most acid-tolerant of the LAB (Kashket, 1987 and Kandler and Weiss, 1986).

Lactobacilli are found in dairy, grain, meat, fish products, water, sewage, beer, wine, fruits and fruit juice, pickled vegetables, sauerkraut, silage, sourdough, and mash. They are a part of the normal flora of mouth, intestinal tract and vagina of human and animals (Hammes, *et al.*, 1995). They are rarely pathogenic. There are reports that lactobacilli, in conjunction with *Streptococcus mutans*, cause dental caries (Rogosa, *et al.*, 1953). *L. casei* subsp. *rhamnosus*, *L. acidophilus*, *L. plantarum* and *L. salivarius* have been found occasionally associated with subacute bacterial endocarditis, systemic septicemia and abscesses.

Lactobacilli cultures are used widely as starters for production of buttermilk, yogurt, cheese, fermented meat and vegetables, sausage, soy sauce and sauerkraut (Damalin, *et al.*, 1995). Lactobacilli have potential for use as probiotics, live-microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance, to prevent or cure a variety of illness, for examples, the prevention of bacterial vaginosis, vulvovaginal candidiasis and urinary tract infections (McGroarty, 1993, McGroarty, 1994 and Fuller, 1989). Some lactobacilli, *L. rhamnosus*, *L. acidophilus*, *L. fermentum*, *L. casei* subspecies *alactosus* and *L. jensenii* produce fimbriae that are responsible for cell attachment to surfaces. They are believed to control the normal flora in their habitat. Lactobacilli may produce bacteriocins (protein or peptide antimicrobials produced by bacteria), which are lethal or inhibitory to other, often closely related, bacteria (Earnshaw, 1992).

Lactobacilli are classified into three groups, based on their carbohydrate fermentation patterns (Table 1), configuration of the lactic acid produced (Table 2), hydrolysis of arginine, growth requirements, growth temperatures, peptidoglycan structure, electrophoretic motility of LDH, mol% G+C of the DNA and DNA-DNA homology studies (Axelsson, 1993, Kandler, 1984, Kandler and Weiss 1986, Hammes, *et al.*, 1995 and Schleifer, *et al.*, 1995).

Table 1. Classification of the Genus *Lactobacillus*.

| Characteristic | Group I | Group II | Group III |
|--------------------------------|--|--|---|
| Hexose fermentation | + | + | + |
| Pentose fermentation | - | + | + |
| CO ₂ from glucose | - | - | + |
| CO ₂ from gluconate | - | + ^a | + ^a |
| FDP aldolase present | + | + | - |
| Phosphoketolase present | - | + ^b | + |
| Members | <i>L. acidophilus</i> | <i>L. casei</i> , <i>L. rhamnosus</i> | <i>L. brevis</i> , <i>L. reuteri</i> |
| | <i>L. delbruckii</i> | <i>L. curvatus</i> , <i>L. paracasei</i> | <i>L. buchneri</i> , <i>L. suebicus</i> |
| | <i>L. helveticus</i> | <i>L. plantarum</i> , <i>L. bif fermentans</i> | <i>L. fermentum</i> , <i>L. fructivorans</i> |
| | <i>L. salivarius</i> | <i>L. sake</i> , <i>L. agilis</i> | <i>L. vaccinostercus</i> |
| | <i>L. animalis</i> , <i>L. avarius</i> | <i>L. pentosus</i> , <i>L. graminis</i> | <i>L. kefir</i> , <i>L. oris</i> |
| | <i>L. ruminis</i> , <i>L. mali</i> | <i>L. alimentarius</i> | <i>L. hilgardii</i> , <i>L. vaginalis</i> |
| | <i>L. sharpeae</i> | <i>L. coryniformis</i> , <i>L. homohiochii</i> | <i>L. parabuchneri</i> , <i>L. sanfrancisco</i> |

^a When fermented. ^b Inducible by pentoses

Table 2. Lactic acid isomers produced by selected lactobacilli (Hammes, *et al.*, 1995 and Severson, 1998)

| Species | Lactate isomer(s) ^a | Type of fermentation | Growth 15/ 45(°C) | Sucrose fermented |
|---|--------------------------------|----------------------|-------------------|-------------------|
| <i>L. acidophilus</i> | D/L | Obligate | +/- | + |
| <i>L. amylophilus</i> | L | Obligate | +/- | - |
| <i>L. amylovorus</i> | D/L | Obligate | +/- | + |
| <i>L. casei</i> ssp. <i>casei</i> | L | Facultative | +/- | + |
| <i>L. casei</i> ssp. <i>rhamnosus</i> | L | Facultative | +/- | + |
| <i>L. curvatus</i> | D/L | Facultative | +/- | +/- |
| <i>L. delbrueckii</i> ssp. <i>bulgaricus</i> | D | Obligate | +/- | - |
| <i>L. delbrueckii</i> ssp. <i>delbrueckii</i> | D | Obligate | +/- | + |
| <i>L. delbrueckii</i> ssp. <i>lactis</i> | D | Obligate | +/- | + |
| <i>L. helveticus</i> | D/L | Obligate | +/- | - |
| <i>L. murinus</i> | L | Facultative | +/- | + |
| <i>L. plantarum</i> | D/L | Facultative | +/- | |
| <i>L. salivarius</i> | L | Obligate | +/- | + |

^a D- or L- isomers make up 90% or more of total; D/L, 25-75% is L(+) lactate

Group I: Obligate homofermenters: The organisms in this group do not possess a phosphoketolase and therefore cannot ferment pentoses. Hexoses are fermented through a homolactic fermentative pathway.

Group II: Facultative heterofermenters: Hexoses are fermented to lactic acid via the Embden Meyerhoff Parnas (EMP) pathway. The bacteria have both an aldolase and a phosphoketolase. In the absence of glucose, they ferment pentoses via a heterolactic fermentative pathway.

Group III: Obligate heterofermenters: These bacteria ferment hexoses via a phosphogluconate pathway, yielding lactate, ethanol, acetic acid and CO₂ in equimolar amounts. Pentoses may be fermented.

Lactobacilli require not only carbohydrates for growth but also specific nucleotides, amino acids and vitamins. Thiamine is necessary for growth of heterofermenters. Pantothenic acid is required for all strains. The requirement for folic acid, pyridoxal phosphate, p-aminobenzoic acid and riboflavin (the most frequently required compound) varies depending on the species. Because of its riboflavin requirement, *Lactobacillus casei* is used in a bioassay for riboflavin (Baker, *et al.*, 1975). Lactobacilli require inorganic nutrients in defined media. Phosphate, potassium and magnesium are absolute requirements. Manganese, but not iron, is required by *L. casei* for their nLDH. Manganese has shown to be a required cofactor for other enzymes produced by the lactobacilli, and it has been linked with aerotolerance in these bacteria (Tseng, *et al.*, 1993). Cobalt and lithium inhibit the conversion of substrate to lactic acid in *L. casei* (Tuli, *et al.*, 1985). In some strains, high levels of phosphate inhibits growth.

Lactobacilli do not contain a cytochrome system for oxidative phosphorylation. They do not have catalase. Some lactobacilli can metabolize the end-product lactate to acetate and CO₂ under aerobic conditions by stereospecific NAD⁺- independent, flavin-containing lactate dehydrogenases (in *L. casei*, *L. plantarum*, *L. coryniformis* and *S. faecium*) or lactate oxidases (in *L. carvatus*, *L. sake*, *L. acidophilus*, *L. bulgaricus* and *L. lactis*) (Kandler, 1983 and Strittmatter, 1959). Lactate oxidase uses O₂ as an electron acceptor to yield pyruvate and

H₂O₂. NAD-Independent, flavin-containing lactate dehydrogenase oxidizes lactate to pyruvate and H₂O₂ in the presence of O₂. Pyruvate accumulates and spontaneously splits into acetate and CO₂. Upon an addition of methylene blue, the oxidation rate increases significantly. Lactobacilli do not have superoxide dismutase to protect themselves from O₂⁻ but they possess a unique manganese-catalyzed scavenging system (Kandler and Weiss, 1986). Lactobacilli can be serologically grouped (from A to G) based on specific antigenic determinants on their cell wall and/or cell membrane (Table 3).

***Lactobacillus casei* subspecies *rharnosus* or *Lactobacillus rharnosus*:** This bacterium produces L- (+) lactic acid by a homofermentative pathway. It contains isoprenoid quinones which are not found in other lactobacilli. These isoprenoids are involved in peptidoglycan and teichoic acid synthesis (Cerning, 1990). *L. casei* subsp. *rharnosus* possesses a capsular, rhamnose-containing typing antigen. The quantity is dependent on cultural conditions. *Lactobacillus casei* does not grow on media lacking valine, glutamate, tryptophan and arginine. It can grow without isoleucine, leucine, serine, tyrosine, aspartate, cysteine and phenylalanine. *L. casei* also does not require alanine, asparagine, glutamine, glycine, lysine, proline, histidine, methionine and threonine (Morishita, *et al.*, 1981). *Lactobacillus casei* is used in production of an exopolysaccharide (EPS) used in fermented milk to prevent syneresis and to ensure proper texture and body. The optimal pH for EPS production is between 6.0-6.5 (Mozzi, *et al.*, 1994). EPS formation is favored by the presence of excess carbohydrate and low temperature. Nitrogen, phosphorus or sulfur limitation also increases EPS formation.

***Rhizopus oryzae*:** *Rhizopus oryzae* is a commercial strain with high potential for L- (+) lactic acid production. It has fewer nutritional requirements than the lactobacilli and can grow on variety of carbohydrates, including starch (Hang, 1989 and Yu, *et al.*, 1989). Disadvantages of this strain include low productivity and yield in comparison to the lactobacilli. *Rhizopus* produces metabolites other than lactic acid; notably glycerol, ethanol and fumaric acid. The product effluent from *rhizopus* lactic acid fermentations contain compounds which

Table 3. Group antigens of lactobacilli (Sharpe, 1981 and Kandler and Weiss, 1986)

| Group | Species | Antigen | Location | Antigen determination |
|-------|--------------------------|------------------------|---------------|-----------------------|
| A | <i>L. helveticus</i> | Glycerol teichoic acid | Cell wall | D-glucosyl |
| B | <i>L. casei</i> | Polysaccharide | Cell wall | L- rhamnosyl |
| C | <i>L. casei</i> | Polysaccharide | Cell wall | D- glucosyl |
| D | <i>L. plantarum</i> | Ribitol teichoic acid | Cell wall | D- glucosyl |
| E | <i>L. delbrueckii</i> | Glycerol teichoic acid | Cell wall | |
| | <i>subsp. lactis</i> | | | |
| | <i>subsp. bulgaricus</i> | | | |
| | <i>L. brevis</i> | | | |
| | <i>L. buchneri</i> | | | |
| F | <i>L. fermentum</i> | Glycerol teichoic acid | Cell membrane | D- galactosyl |
| G | <i>L. salivarius</i> | Polysaccharide | Cell wall | L- rhamnosyl |

can produce recovery problems (Soccol, *et al.*, 1994 and Yang, *et al.*, 1995). *Rhizopus* fermentations produce high viscosity broths because of mycelial aggregation. Numerous approaches, including cell immobilization, have been tried to resolve these problems (Dong, *et al.*, 1996 and Hamamci, *et al.*, 1994).

Bacillaceae family: Recently, other lactic acid producing bacteria have been investigated for commercial production. Bacteria in the family of Bacillaceae capture most attention. *Bacillus* species are rod shaped Gram positive, aerobic spore formers. *Bacillus coagulans* and *Bacillus stearothermophilus* have been evaluated for commercial lactic acid production. These strains produce L- (+) lactic acid via a homolactic fermentative pathway, at high temperatures (between 54 to 60°C for *B. coagulans* and 65°C for *B. stearothermophilus*) and at a high sugar concentration (140 to 190 g/l for *B. coagulans*). These bacteria require complex nutrients, as do the lactobacilli. However, they can favorably compete with conventional strains due to their tolerance for high temperature and substrate concentration (Severson, 1998, Danner, *et al.*, 1998 and Fritze, *et al.*, 1992). Another bacterial strain with potential to become a commercial lactic acid producer is *Sporolactobacillus inulinus* which produces D- (-) lactic acid via a homofermentative pathway. This bacterium forms a single endospore and is catalase negative. It grows best in microaerophilic conditions and can completely ferment between 20 and 30% glucose to D- (-) lactic acid (Gibson, *et al.*, 1974).

B.2 Carbohydrate metabolism of the lactobacilli:

B.2.1 Homolactic fermentation. Homolactic fermentation utilizes the Embden-Meyerhof Parnas (EMP) pathway for glycolysis where glucose is metabolized to fructose-1,6-diphosphate (FDP). FDP is split by the enzyme FDP aldolase into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate. Both metabolic intermediates are ultimately converted into pyruvic acid during the metabolic sequence which includes substrate level phosphorylation at two sites, involving a phosphoglycerate kinase and a pyruvate kinase. In a homolactic fermentation, the key step is the conversion of pyruvate to lactic acid by an NAD⁺-dependent lactate dehydrogenase (LDH) and the regeneration of the nicotinamide

adenine dinucleotide (NAD^+) which is recycled to the glycolytic pathway, maintaining a redox balance. Lactic acid is the sole end product. In a homolactic fermentation, one mole of glucose is oxidized to two moles of pyruvic acid, which are ultimately converted to two moles of lactic acid. Two moles of ATP are utilized and four moles are produced via substrate level phosphorylation. That is a net gain of two moles of ATP (Fig. 6) (Neidhardt, *et al.*, 1990, Holzappel, *et al.*, 1995, and Garvie, 1980).

B.2.2 Heterolactic fermentation: In a heterolactic fermentation, the 6-phosphogluconate, pentose phosphate pathway or hexose monophosphate shunt, is used. Ethanol and CO_2 are produced in addition to lactic acid. In this metabolic pathway, glucose is phosphorylated by an enzyme, glucokinase, yielding glucose 6-phosphate, which is subsequently dehydrogenated by a glucose 6-phosphate dehydrogenase using NAD^+ as a cofactor, producing 6-phosphogluconate. A 6-phosphogluconate dehydrogenase then decarboxylates 6-phosphogluconate in the presence of NAD^+ yielding ribulose 5-phosphate. The ribulose 5-phosphate is epimerized to xylulose 5-phosphate, which is cleaved into glyceraldehyde 3-phosphate (which is further metabolized as in the EMP pathway producing lactic acid) and acetyl phosphate, which is reduced to ethanol by the enzymes phosphotransacetylase, acetylaldehyde-CoA dehydrogenase and alcohol dehydrogenase. The end products are lactic acid, ethanol and CO_2 (Fig. 7). This metabolic pathway yields only one mole of lactic acid for each mole of glucose, and provides the bacterial cell a net gain of one mole of ATP (Axelsson, 1993).

Some strains of lactobacilli can change their metabolic pattern, by unknown mechanisms, from homofermentative to heterofermentative depending on the limitations of substrate and oxygen. Pyruvate can have many alternative fates, besides being converted to lactic acid, depending on the substrate availability. Pyruvate can be reduced to lactate, CO_2 , alcohol, formate and acetate (Fig. 8). The organisms carrying out heterolactic fermentation achieve nutritional advantages by adjusting the relative amounts of each end product. They

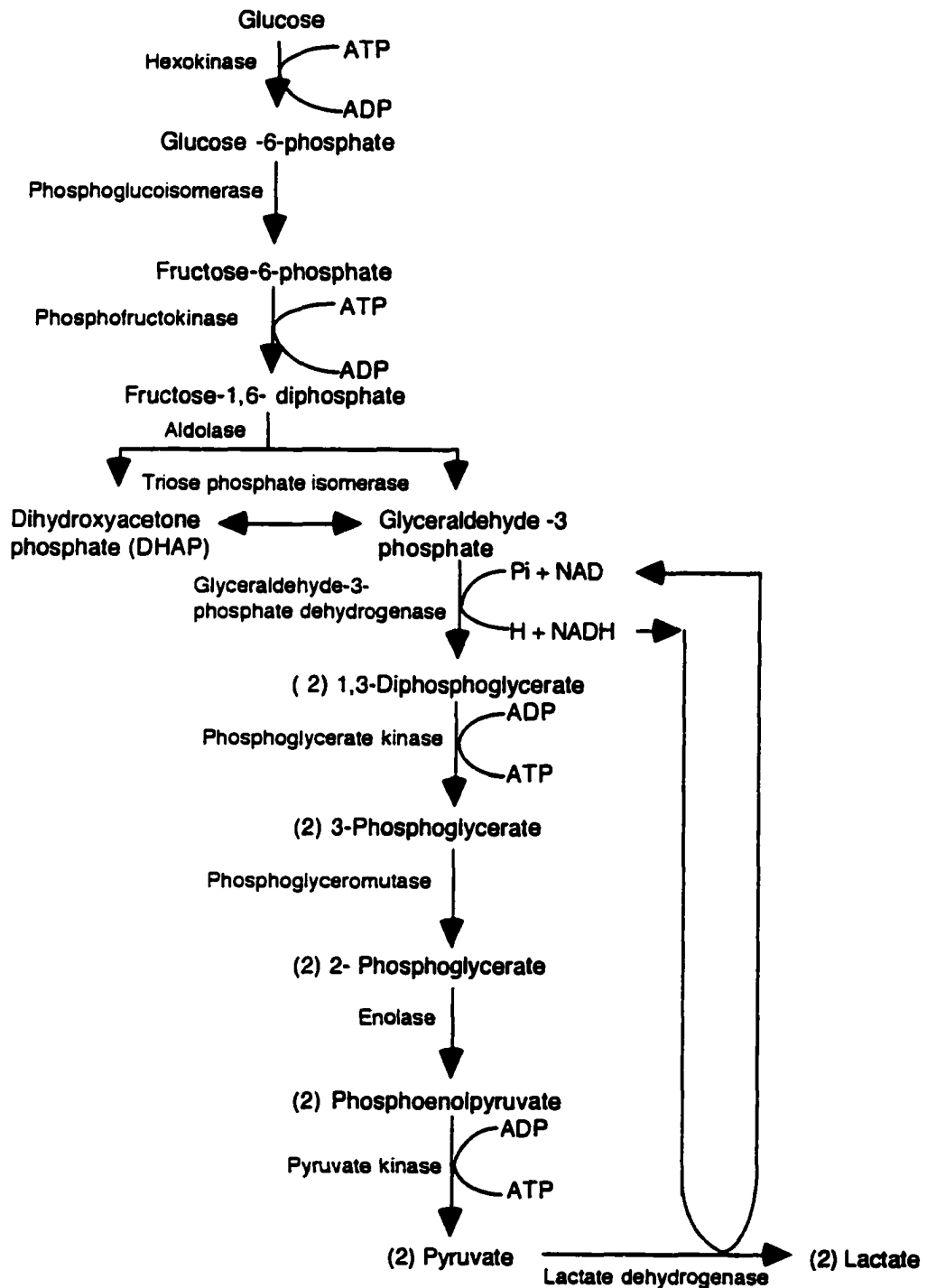


Figure 6. The homofermentative pathway.

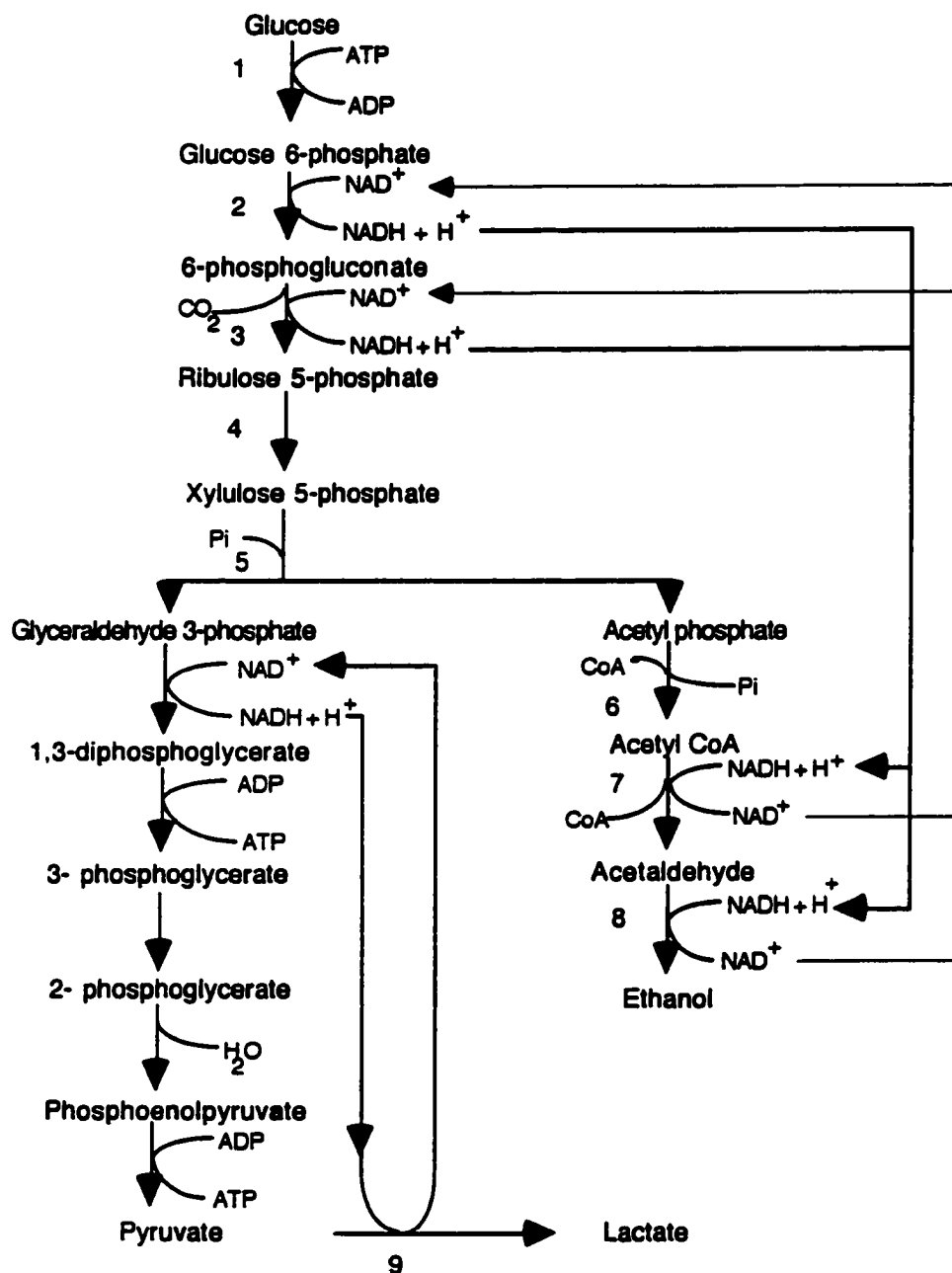


Figure 7. The 6-phosphogluconate pathway. Selected enzymes are numbered: 1. Glucokinase; 2. Glucose 6-phosphate dehydrogenase; 3. 6-phosphogluconate dehydrogenase; 4. Epimerase; 5. Phosphoketolase; 6. Phosphotransacetylase; 7. Acetaldehyde-CoA dehydrogenase; 8. Alcohol dehydrogenase; 9. Lactate dehydrogenase.

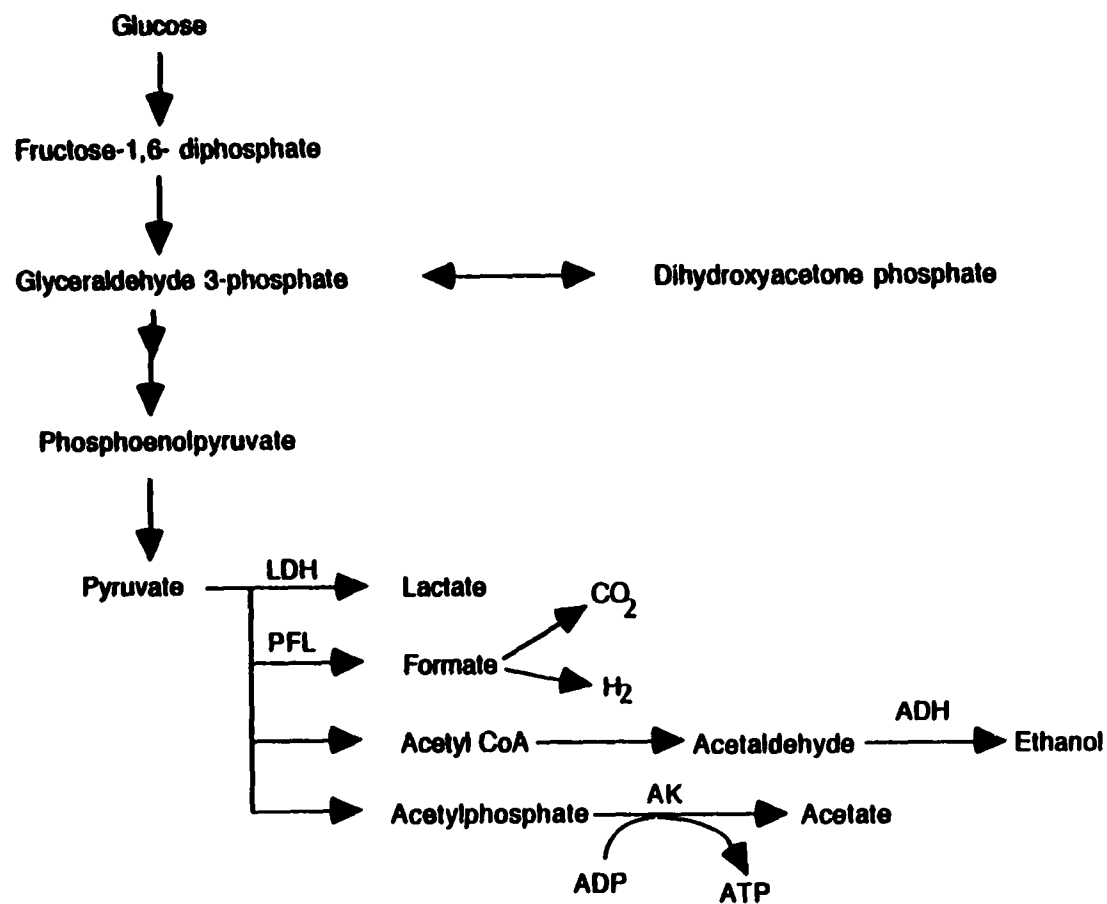


Figure 8. The alternative fates of pyruvate from some lactobacilli. Lactobacilli may change their metabolisms from homofermentative to heterofermentative metabolic pathways based on the availability of substrates. LDH, Lactate dehydrogenase; PFL, pyruvate formate lyase; ADH, alcohol dehydrogenase; AK, acetate kinase

can usually grow anaerobically on reduced substrates such as sorbitol, or can live on more oxidized substrates such as glucuronic acid (Neidhardt, *et al.*, 1990).

B.3 Lactic acid configuration and lactate dehydrogenase: The lactic acid isomer produced by a given organism is dependent on the specific NAD⁺-lactate dehydrogenase (nLDH) present. Some bacteria contain both a D- and an L-nLDH, in equivalent amounts, while many organisms produce only one form of the enzyme. *L. curvatus* and *L. sake* can convert L- (+) lactic acid to D- (-) lactic acid using a racemase which is induced by L- (+) lactic acid (Garvie, 1980). For organisms that produce both D- and L- lactic acid, L- (+) lactic acid is generally produced early in the growth while D- (-) lactate is produced in the late log to stationary phases.

Lactate dehydrogenase is a key enzyme for energy production by lactic acid bacteria. It irreversibly converts pyruvate to lactate. There are two types of LDH, NAD⁺-dependent and NAD⁺-independent (nLDH and iLDH). The active nLDH molecule is a tetramer with a molecular weight of 120,000 - 140,000. NAD⁺-dependent lactate dehydrogenase is cytoplasmic. *L. casei* possesses an allosteric L- nLDH which is activated by FDP and Mn²⁺. In most cases, D- nLDH is reversible. Some DL- lactic acid forming bacteria produce L-nLDH's which do not require FDP, and catalyze reversible reactions. Generally, inorganic phosphate inhibits the activity of FDP-dependent L-nLDH. At pH 4.5 or lower, FDP has no effect on the nLDH of *L. casei* (Garvie, 1980).

B.4 Solute transport

B.4.1 PMF-driven (active or secondary) transport: Proton motive force (PMF) is generated by an electrical potential (inside negative) and a pH gradient (inside alkaline) across the cytoplasmic membrane. The PMF generated is large enough to be converted into chemical energy as ATP. In aerobes, this is the function of ATP synthase (ATPase or H⁺ATPase). LAB do not possess electron transport chains, therefore, they cannot generate ATP by this method. In LAB, ATP is generated only by substrate level phosphorylation. However, LAB have ATP synthase-like enzymes which hydrolyze ATP and

concomitantly pump protons across cell membranes, establishing a PMF (Konings, *et al.*, 1989 and Maloney, 1990). The ATPase of *L. casei* has the same basic structure as mitochondrial and chloroplast ATPase's (Muntyan, *et al.*, 1990). Lactobacilli can tolerate intracellular pH as low as 4.2-4.4. The extrusion of protons by ATPase and the electrogenic uptake of K⁺ maintain an interior more alkaline than the surrounding media.

In PMF- driven transport, ATPase pumps out protons at the expense of ATP to create a proton gradient. A solute is translocated to the cytoplasm in a symport fashion with a proton through a specific membrane-associated protein (carrier or permease). The process is reversible. Intracellular solutes (or products) are driven outwardly with protons through the proton symport resulting in PMF formation (Fig. 9). It is believed that lactate efflux uses this mechanism. In order to maintain an electrogenic efflux, more than one proton has to be exported per molecule of lactate. When the external pH is too low or the extracellular lactate concentration is too high, PMF ceases. The PMF-driven transport also controls acetate efflux in *L. plantarum*. Many sugars are transported in a similar manner. It has been suggested that the heterofermenters use exclusively PMF-driven sugar transport, since no sugar PTS system has been found in these organisms (Axellsson, 1993).

B.4.2 Substrate/product antiport (exchange transport): In LAB substrate/product antiport is common. For example, lactose import is coupled with galactose efflux in an antiport-like system. For arginine fermenters such as *Streptococcus lactis*, arginine, which is metabolized simultaneously with carbohydrate, is taken up in exchange of ornithine, which is excreted into the medium (Driessen, 1987).

B.4.3 Phosphate-bond-linked transport: In LAB, this process is irreversible and PMF-independent. It has been reported that when the internal ATP is low, the transport system decreases. This suggests a direct involvement of ATP and/or another high energy compound (Poolman, *et al.*, 1987). Glutamate and glutamine transports in lactococci use this system. The mechanism prefers glutamic acid, which leads to growth limitations at alkaline pH values. Glutamine transport is pH independent.

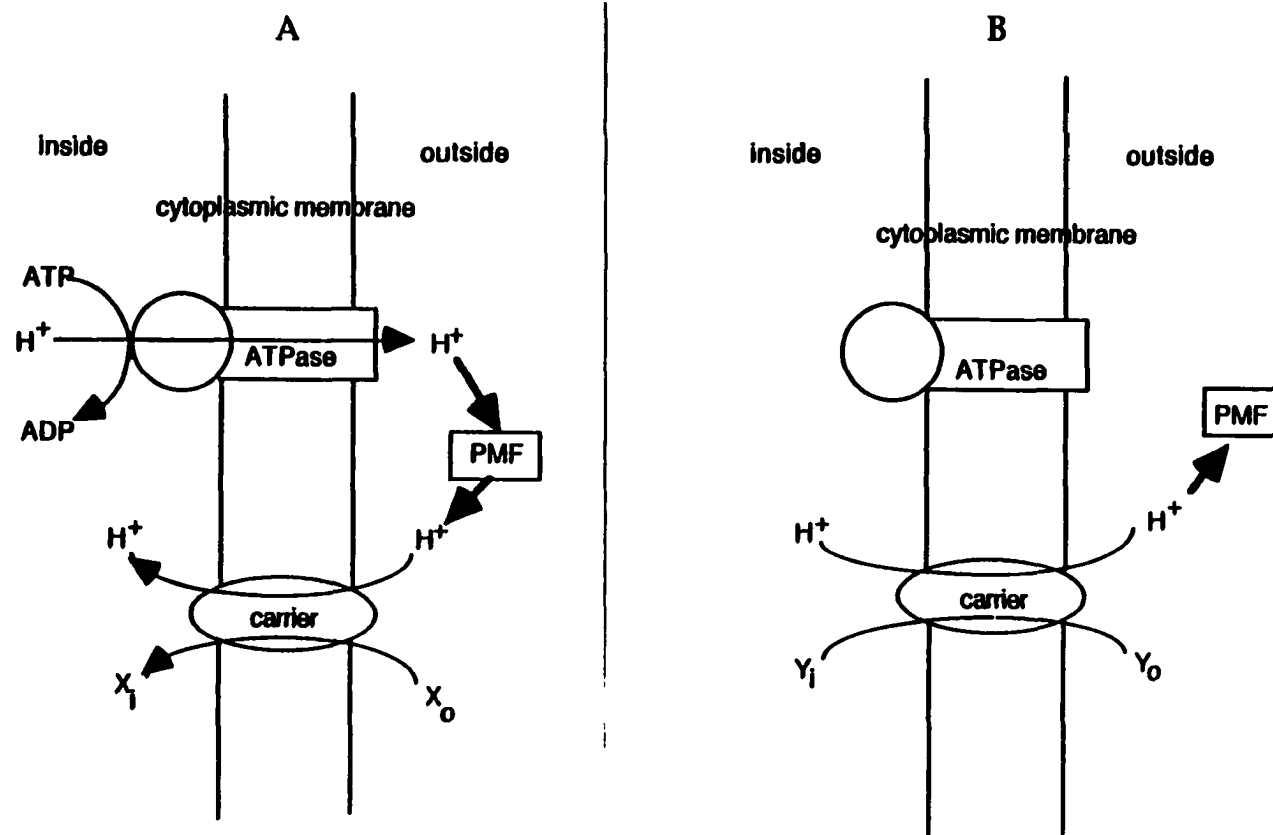


Figure 9. Proton motive force (PMF) formation. A, by ATPase and PMF-driven transport. B, electrogenic end-product efflux

B.4.4 Group translocation or the phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS): The homofermenters use either a PTS system or group translocation to import carbohydrates (Axelsson, 1993). The sugar is phosphorylated after transportation. The high energy phosphate bond of PEP provides the energy for the process. The system is composed of sugar-nonspecific proteins (Enzyme I and Heat-stable protein, HPr) and sugar-specific proteins (Enzyme II and III). E II is a membrane-located enzyme and acts in concert with phosphorylated E III to mediate recognition, translocation and phosphorylation of sugars. The system is closely linked with glycolysis (Fig. 10). During starvation, FDP is scarce, PK and PTS are inoperative. The cell contains high concentrations of PEP, 3-phosphoglycerate and 2-phosphoglycerate. This metabolite pool is called the "PEP potential," which enables the cell to rapidly resume transport and glycolysis once a sugar becomes available.

Sugar influx: In LAB, simple carbohydrates enter the major catabolic pathways at the level of glucose 6-phosphate or fructose 6-phosphate, except for galactose, which uses a PEP: PTS system to enter the cell. Galactose is phosphorylated to galactose 6-phosphate that is further metabolized through the tagatose pathway (Bissett, *et al.*, 1974 and Bettenbrock, *et al.*, 1999). Tagatose is a fructose stereoisomer that requires different enzymes for metabolism. Galactose 6-phosphate is metabolized to tagatose 6-phosphate by an enzyme tagatose 6-phosphate isomerase. An enzyme tagatose 6-phosphate kinase then phosphorylates the tagatose 6-phosphate to yield tagatose 1,6-diphosphate, which is further metabolized into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by an enzyme tagatose 1,6-diphosphate aldolase. Galactose can enter a cell by a specific permease and is then metabolized via the Leloir pathway (Fig. 11). The enzymes in the Leloir and the tagatose 6-phosphate pathways are different. In lactobacilli, there is evidence that the bacteria utilize galactose via both pathways.

Lactose can be taken up using either a permease or the PEP: lactose PTS system. *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis* and *L. acidophilus* use lactose

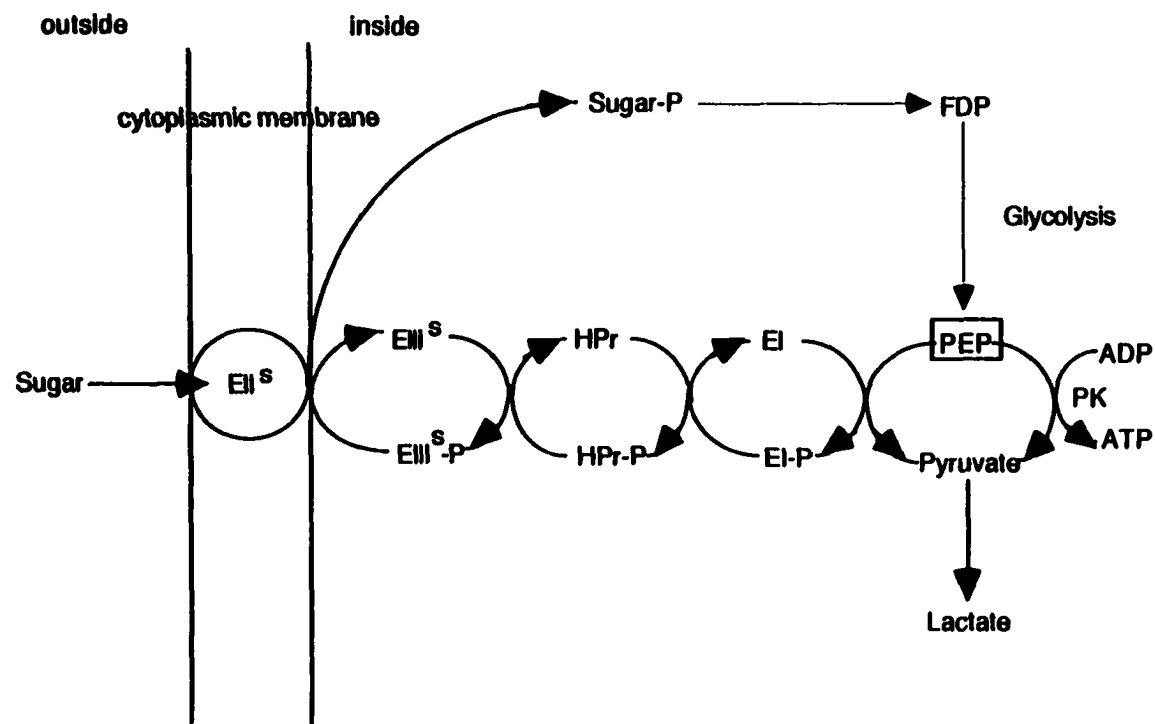


Figure 10. The phosphoenolpyruvate: sugar-phosphotransferase system for sugar transport (PEP:sugar-PTS). s, specific sugar; P, phosphate; PK, pyruvate kinase; HPr, heat-stable protein

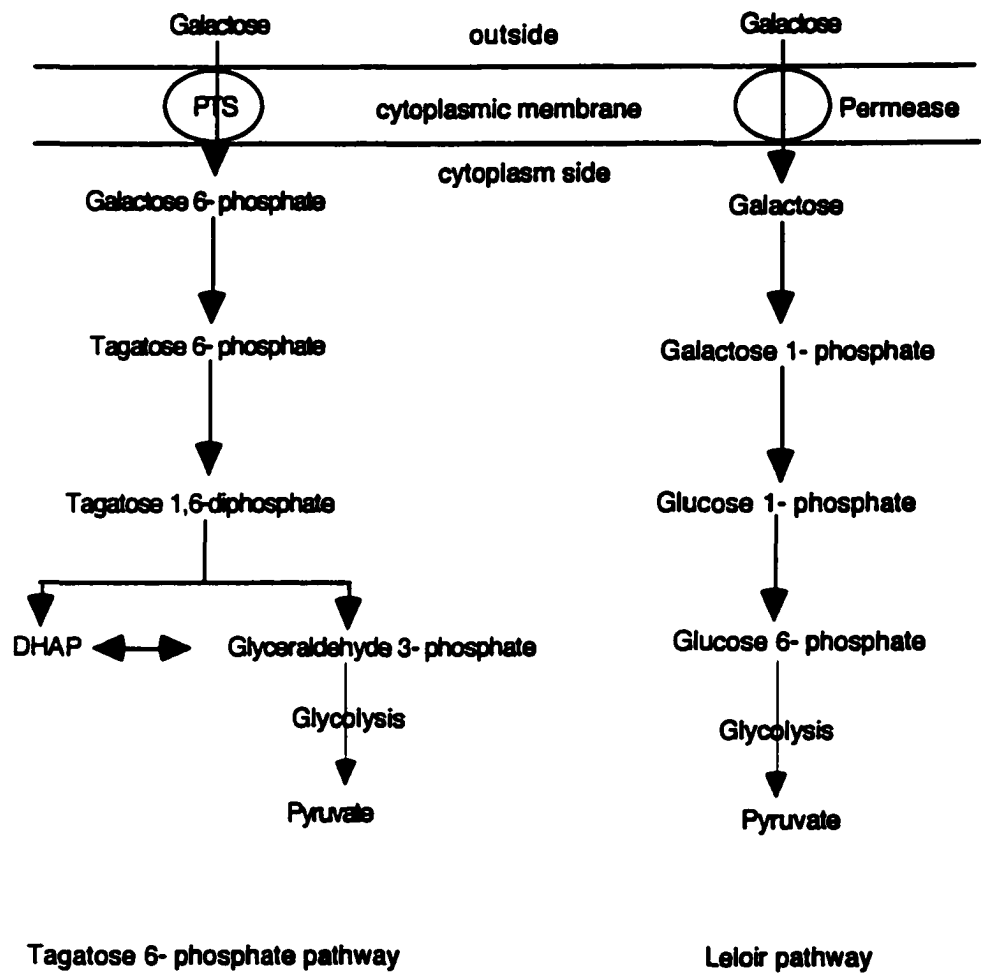


Figure 11. The tagatose 6- phosphate and the Leloir pathways in lactic acid bacteria.

permease to take up lactose which is further metabolized by β -galactosidase, yielding glucose and galactose. Glucose is metabolized while galactose is excreted. This maybe due to either low β -galactokinase activity or possibly an energetically favorable reaction of the lactose transport system. *L. casei* takes up lactose by either PEP: lactose PTS system or a permease. By the PEP: lactose PTS system, upon entering the cell, lactose is phosphorylated to lactose 6-phosphate. The lactose 6-phosphate is split by phospho- β -D-galactosidase to give glucose and galactose-6-phosphate. Glucose enters glycolysis while galactose 6-phosphate goes to the tagatose pathway. The PEP: lactose PTS system is induced or repressed by glucose (Kandler, 1983 and Axelsson, 1993). In *Lactococcus lactis*, *Staphylococcus aureus* and *Streptococcus mutans*, the genes coding for the enzymes in the tagatose 6-phosphate pathway are in part of the *lac* operon, while those genes in *L. casei* are not (Bettenbrock, *et al.*, 1999). Similar to other sugar transports, sucrose permease catalyzes sucrose transport into the cell, where a specific sucrose hydrolase splits the sugar to glucose and fructose before it enters further pathways. Sucrose induces, and is cleaved by, a specific sucrose 6-phosphohydrolase to yield glucose 6-phosphate and fructose. Sucrose also serves as a donor of monosaccharides for exopolysaccharide formation (Thompson, *et al.*, 1981).

Lactate efflux: In homofermenters, lactate is exported by using PMF as described above. In *L. helveticus*, an electroneutral transport (simple diffusion) has been observed. The system is carrier-mediated. This bacterium produces large amount of lactic acid when the external pH is below 4.0. The mechanism is unknown (Gatje, *et al.*, 1991).

B.5 Regulation of lactic acid synthesis: The regulation of lactic acid production appears to be at the enzyme level. The key enzyme in the homofermentative pathway is lactate dehydrogenase. There are two forms of the enzyme, based on its effectors, FDP-dependent and FDP-independent. The FDP-dependent L-nLDH is found only in Gram positive bacteria, and exists normally as an inactive monomer or dimer (Mayr, *et al.*, 1980). Binding of FDP induces a conformational change in the L-nLDH, thus, activating this allosteric enzyme. Allosteric change decreases the K_m for pyruvate, and sometimes for NADH (Mayr, *et*

al., 1982, Crow, *et al.*, 1977 and Russel, *et al.*, 1996). Normally, the optimal pH of L-nLDH is less than 5.5. At low pH, FDP is not required, as protons drive tetramer formation, the active form of L- nLDH. Besides FDP, Co^{2+} and Mn^{2+} induce and activate the enzyme by an unknown mechanism. Free inorganic phosphate increases the requirement for FDP and it appears to be a competitive inhibitor of FDP binding (Fig. 12). For the FDP-independent L-nLDH, it has been reported that the enzyme subunits are permanently bound together as active tetramers. The FDP-independent L-nLDH from *S. bovis* appeared to be stabilized by phosphate although the activity of the enzyme was also reported to be inhibited by phosphate (Yamada and Carlsson, 1975).

One of key enzymes of glycolysis, pyruvate kinase (PK), also plays a role in controlling the homofermentative pathway. The enzyme is activated by FDP, glucose 6-phosphate and/or galactose 6-phosphate, and is inhibited by intracellular phosphate. Thus, when there is an abundance of substrate, FDP is plentiful and both PK and L-nLDH are activated, resulting in high production of lactic acid. However, when substrate is scarce, FDP is low and inorganic phosphate is abundant. PK is inactivated. L-nLDH is in an inactive form, and no lactic acid is produced (Russel, 1996).

In heterolactic fermenters, the enzyme pyruvate formate lyase (PFL) plays a role in lactate production. The enzyme is sensitive to an intracellular pH lower than 7.5. Triose phosphate, glyceraldehyde 3-phosphate and DHAP inhibit the action of PFL. At low substrate levels, where there is no FDP to activate L-nLDH, pyruvate is metabolized by PFL yielding acetyl CoA and formate. NADH is oxidized by alcohol dehydrogenase. When substrate is abundant, the bacteria prefer to use the LDH to the PFL system.

Another factor involved in lactic acid regulation is pH. At low extracellular pH's, LAB decrease their intracellular pH producing a small pH gradient to prevent undissociated lactic acid from accumulating inside the cells. PFL is shut down while nLDH is activated (Cook, *et al.*, 1994).

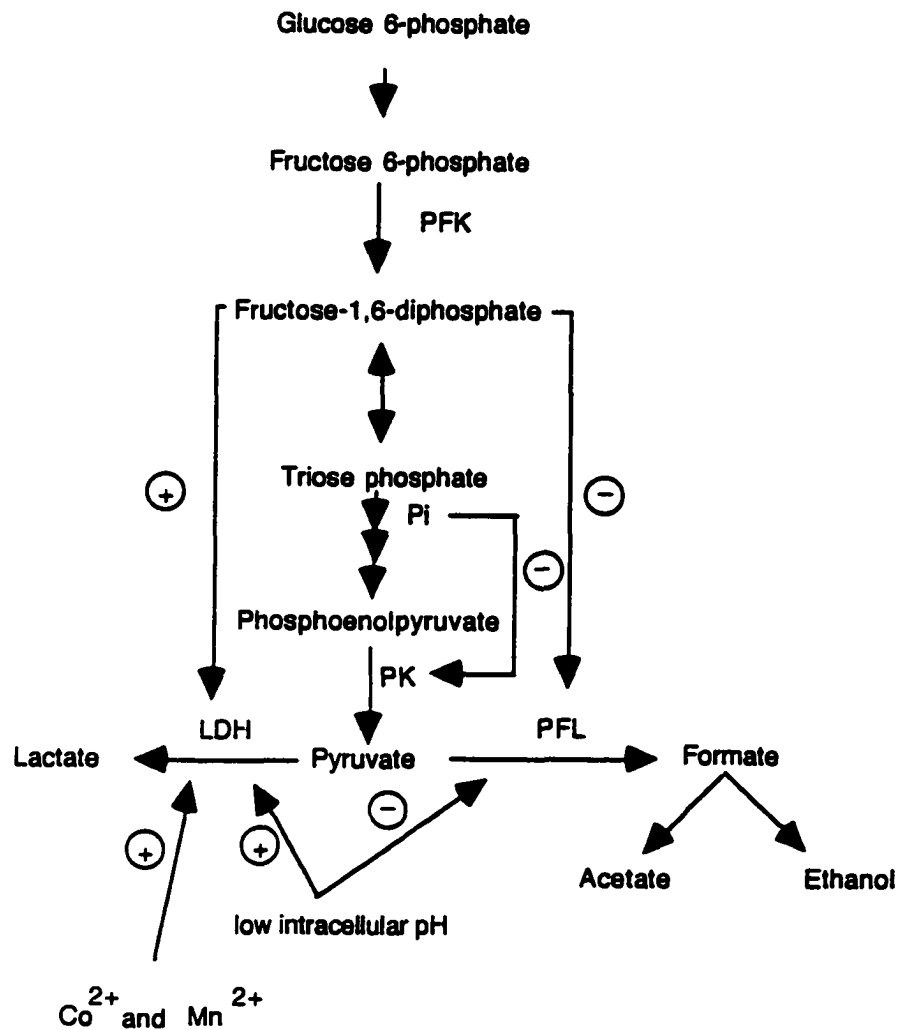


Figure 12. The regulation of lactic acid production in LAB. Pi, phosphate; PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase.

In *S. bovis* (a member of LAB), proton motive force (PMF) is created by the F_1F_0 ATPase, which is stimulated by FDP. PMF is used in solute transport, including lactate efflux. The F_1F_0 ATPase has a relatively high affinity for ATP and is inhibited by a proton gradient. During starvation for nitrogen or treatment with antibiotics, such as chloramphenicol, to inhibit protein synthesis, cells produce ATP by glycolysis at a higher rate than the rate of ATP utilization. ATP accumulates and is metabolized by non-growth mechanisms, while lactic acid production increases (Russel, 1996).

IV. Industrial Processes for Lactic Acid Fermentation

A. Batch fermentation: Batch fermentation has been used to produce lactic acid since production first began in the 1890's. An appropriate carbohydrate and suitable complex nitrogen source to supply amino acids, vitamins and mineral salts are batch loaded into the fermenter. Normally, the sugar concentration in the medium is between 5 and 12%. The inoculation size is approximately 5 to 10% v/v. The temperature is maintained, depending on bacterial strain, between 38 to 46°C. The fermentation pH is controlled between 5.4 to 6.4, depending on bacterial strain, by adding a caustic, such as calcium hydroxide, sodium hydroxide, ammonium hydroxide or calcium carbonate. The fermentation is allowed to continue until the utilizable carbohydrate is exhausted, usually between 5 and 10 days. Commercial fermentation yields are 93 to 95%. The disadvantages of the lactic acid production by this method are batch to batch variation, significant amount of many waste products, such as gypsum and cell mass, low cell density in the fermenter and low productivity. These factors increase production cost of lactic acid (Tejayadi, *et al.*, 1995).

Continuous processes have been investigated for many years with the goal of improving productivity and reducing the need for expensive complex growth factors, but they are not yet used commercially (Severson, 1998).

B. Substrate sources: The carbohydrate sources are generally agricultural by-products such as; whey, whey permeate, sugarcane molasses, beet molasses, scampi waste (Zakaria, *et al.*, 1998) and corn starch and potato starch, which are selected primarily because

of low cost, availability and relative purity (Datta, *et al.*, 1995). Whey is a by-product of cheese manufacture, and is composed of 65% lactose, 12% protein, 11% inorganics, 0.2% lactic acid and vitamins. Whey permeate is a by-product of the production of whey protein concentrate (Bailey, *et al.*, 1988). It contains higher lactic acid content (approximately 4.5%) than in whey. Corn starch hydrolysate, which is composed of 95% glucose, 1% maltose, 1.2% isomaltose, 1.3% other disaccharides and 2.5% higher saccharides, is the most pure carbohydrate source used for lactic acid fermentations. However, it requires significant nutrient supplementation. For composition of molasses, see section VIII.

LAB have complex nutritional requirement, especially for B vitamins. The complex nitrogen sources are normally derived from additives such as corn steep liquor, yeast extract or soy hydrolysates. Yeast extract is the best nutrient supplement as it is richest in vitamins besides its high purity (Yoo, *et al.*, 1997).

V. Product Recovery

The recovery of lactic acid from the fermentation broth in lactic acid manufacture has caused more problems than the fermentation (Miall, 1978). The fermentation broth contains impurities from both the carbohydrates and nutrient supplements. The market for lactic acid application in polymer industries requires highly purified and heat-stable (exhibits no discolorization on heating to 200°C) isomeric lactic acid, making the recovery of pure lactic acid from fermentation broth a concern (Severson, 1998).

The traditional lactic acid recovery process begins with acidification of the fermentation broth, with sulfuric acid, to free lactate from calcium salts, if calcium carbonate is used as a caustic agent. Calcium reacts with sulfuric acid to form calcium sulfate dihydrate (gypsum) which is precipitated and removed together with cell mass and other suspended solids by filtration. The filtrate is evaporated to yield a technical grade lactic acid with concentration of approximately 35 to 40 %.

Food-grade lactic acid is produced from a fermentation using a cleaner sugar source, with a minimal protein content. The filtrate is treated with activated carbon, evaporated to 25%

solids, retreated with activated carbon and finally evaporated to about 50-65% lactic acid. Sometimes ferrocyanide is used to precipitate iron or copper ions when the product is discolored.

Plastic grade lactic acid can be obtained by esterification at 100°C, for 1 to 4 hours, of the crude lactic acid with methanol, with small amount of sulfuric acid added, to form methyl lactate. Methyl lactate [CH₃CH(OH)COOCH₃] has a boiling point of 144 to 145°C, and is colorless. Purified lactic acid, of high purity and heat-stability, is obtained by distillation and hydrolyzation of methyl lactate. The methanol is recycled (Datta, et al., 1995 and Atkinson and Mavituna, 1983).

VI. Alternate Technologies for Lactic Acid Fermentation and Purification

Many improvements remain to be made to improve conventional lactic acid fermentation. For example, lactic acid is an end-product inhibitor of bacterial growth. The mechanism of growth inhibition by lactic acid is not well understood. It has been proposed that in high lactic acid environment, the non-dissociated form of lactic acid is soluble and accumulates in the cytoplasmic membrane along with the ionized-insoluble form. The cytoplasmic membrane becomes acidic leading to the collapse of the proton motive force, blocking solute transport. The ATP produced is then not used in growth, but for the maintenance of pH homeostasis (Goncalves, *et al.*, 1997). Lactic acid must be removed from the fermenter during the fermentation to decrease this inhibitory effect and increase productivity. Increasing cell mass in the fermenter can also improve lactic acid productivity.

Cell immobilization and cell recycling provide high numbers of cells with the possibility of reusing the microbial catalysts, leading to higher productivity. High cell density in a fermenter allows the desired microorganisms to successfully compete with contaminants, providing an opportunity for continuous fermentation. Many investigators have studied cell immobilization on solid supports for lactic acid production, but none have yet been used commercially. Solid supports used in the cell immobilization studies range widely, including sugar cane pressmud, porous alumina beads, activated charcoal, polyurethane foam,

polyacrylate saddle shaped support, plastic composite support and filter membranes (Iwasaki, *et al.*, 1992, Ho, *et al.*, 1997, Nakano, *et al.*, 1996, Zhu, *et al.*, 1994, Yang, *et al.*, 1992 and Xavier, *et al.*, 1994). Cells can be trapped in gels such as calcium alginate or polyacrylamide but there is evidence that nutrients do not diffuse readily through such beads, leading to lower productivity (Wang, *et al.*, 1995).

Membrane filtration has been used in conjunction with a bioreactor to allow recycling of cells back to the reactor as the product is separated from the culture broth. In 1996, Jeantet, *et al.* produced lactic acid in a bioreactor coupled with a nanofiltration membrane, a pressure-driven membrane process with a molecular weight cut-off situated between reverse osmosis and ultrafiltration. In 1995, Tejayadi, *et al.* used a membrane-based cell-recycling bioreactor to continuously produce lactic acid. The membrane modules were polysulfone hollow fibers with a molecular weight cut-off of 30,000. Lactate was separated in the permeate from the fermentation broth. With this process, lactic acid production rose to 22.5 g/l-hr, with lactic acid concentrations of 89 g/l and a yield of 89%.

Electrodialysis fermentation (ED-F) has also been studied for lactic acid production. The procedure has been used in desalting of sea water, the recovery of nickel and the water treatment in electroplating factories. ED-F method continuously removes lactic acid from the fermentation broth by using an electrodialyzer in combination with ion-exchange membranes. Lactate ion penetrates the anion-exchange membrane and is excluded, while the fermentation broth flows back to the fermenter. However, cells accumulate on the membrane resulting in membrane fouling. Therefore, the process has not been applied in large scale lactic acid production (Hongo, *et al.*, 1986).

Ion exchange chromatography has been used in lactic acid purification a method for removal of end product from the fermenter. Ion exchange provides better recovery of a more highly purified lactic acid compared to the more conventional methods (Vaccari, *et al.*, 1993).

VII. Cell Immobilization

Lactobacilli form biofilms. They are involved in film formation in dental carries and bacterial endocarditis. They are normal flora in human and animal gastrointestinal tracts. Bacterial adhesion depends, in part, on the surface properties of both the bacteria and the solid support. The adhesion is partly due to a hydrophobic interaction between them. The surface of the lactobacilli is hydrophilic, partly due to the lipoteichoic acid constituent in the cell walls. They are mildly negatively charged at alkaline pH's due to cell wall constituents, such as proteins, phosphate and carboxylate groups (Pelletier, *et al.*, 1997). In addition, some lactobacilli, especially for those found in dairy industries, such as *L. casei* subsp. *casei*, *L. paracasei* subsp. *paracasei* and *L. casei* subsp. *rhamnosus* produce exopolysaccharides (EPS) in high sugar environments. The EPS may help the bacteria to anchor to solid supports.

Immobilized cell bioreactors have been investigated as replacement for conventional batch fermentation, as they produce higher cell density, resulting in higher productivity. There are many ways to immobilize cells, and some of them are described below (Mattiasson, 1983). No single technique has a clear advantage over any other, rather use is application dependent.

A. Covalent coupling and cross-linking: This is a good method for enzyme immobilization. For whole cell immobilization, studies have focused on dead cells or cells that are to be utilized for single step conversions only, as live cells divide and leak enzyme. Some of the coupling agents that have been used are carbodiimide, glutaraldehyde and hydroxylalkyl methacrylate.

B. Affinity (biospecific) immobilization: This method has been adopted from affinity chromatography. The support must contain a structure capable of interacting with structures on the cell surface. This application has been used in a blood treatment where "ghost" red blood cells, containing urease or glutaminase, are immobilized on Concanavalin-A Sephadex G-25 and packed in a column.

C. Adsorption: It is useful for large-scale processes. Multipoints for cell binding are observed. Cells strongly stick to the supports and are not easily dissociated from the sorbent. Some parameters must be met for good adsorption, for example, the charge and age of cells, the relationship between volumetric cells and surface area, and the composition of the support and its pore size must be right. The adsorption is believed to occur via hydrophobic and charged interactions between surfaces. A good support should have low flow resistance so that nutrients can pass through readily. Synthetic materials like fibrous fiber of poly vinyl alcohol (PVA) which adsorbs enzymes by electrostatic attraction, and porous alumina beads are good for their ability to withstand harsh conditions, prolonging the life span of the immobilized column. This application has been used widely in waste water treatment, ethanol production and cell mass production, with fritted glass, activated carbon, porous glass, wood chips, controlled pore glass and modified cellulose used as solid supports.

D. Entrapment: Cells are confined in a three dimensional gel lattice. The gel compartments have pores to allow substrates and products to migrate freely. Many synthetic polymers can be used to form gel lattices to entrap cells, some examples are; acrylamide, polyurethane, collagen, agar and agarose, cellulose, alginate and K-carrageenan. This method has been in industrial use for a long time, but has not been popular because of practical problems. For example, when entrapped cells divide, they eventually break the lattice. Mass cannot be transferred readily across the compartments causing nutrient starvation to the inner cells, which eventually changes the pattern of production. Calcium alginate beads shrink, and calcium ions are displaced by lactate ions during fermentation causing disruption of immobilization and production patterns (Demirci, *et al.*, 1995, Eikmeier, *et al.*, 1987 and Roy, *et al.*, 1987). The cost of cell entrapment in gels is usually high (Mattiasson, 1983 and Ho, *et al.*, 1997).

VIII. Molasses

Blackstrap or final molasses is the final mother liquor from the crystallization of sucrose at raw sugar mills. This final molasses is the liquid residue from which no more sugar can be

removed economically in the trade (Ruter, 1975 and Meade, *et al.*, 1977). Blackstrap molasses is heavy and viscous. It is inedible and not used for human consumption. The typical composition of blackstrap molasses is shown in Table 4. Sucrose, glucose and fructose are the primary sugars present. Other simple carbohydrates such as mannose and psicose have been reported in small amounts. The principle ash in molasses is in carbonate form. Potash is found to be approximately 40% of the total ash in molasses, while lime and sulfates are 10 to 20%. Other ash components that are found in molasses are magnesium salts, silica, chlorides, phosphates, aluminum, sodium, and iron oxides.

Nitrogenous compounds in molasses are mainly crude protein and some amino acids, aspartic, glutamic acids and asparagine predominate. Waxes, sterols and phosphatides are found, as well as such vitamins as thiamine, riboflavin, niacin, pantothenic acid and biotin. The process for manufacturing raw cane sugar and producing molasses is as follows (Meade, *et al.*, 1977):

1. **Sucrose extraction** After sugar cane is harvested and transferred to sugar mills, the cane stalks are cut into chips with revolving knives. Using heavily grooved crusher rolls to break the cane or a diffuser, a large part of cane juice is expressed. Practically, 95% of the sucrose is extracted from the cane stalks.

2. **Purification of juice (clarification)** The colored raw juice is turbid and acidic. Some soluble and the insoluble impurities must be removed. Approximately 1 lb of CaO is used to neutralize the acidity in the juice from a ton of cane. Albumin, fats, waxes and gums are precipitated and entrapped in suspended solids and finer particles by heating the limed juice. Clear juice is separated from the mud by sedimentation and filtration. The press cake is discarded or returned to the field for fertilizer while the clear juice goes to multiple-effect evaporators.

3. **Evaporation** The clear juice contains about 15% solids. About two-thirds of the water is evaporated under vacuum from the juice to make a syrup, which contains approximately 65% solids and 35% water.

Table 4 . Composition of blackstrap molassas

| Components | Average percentage |
|---|--------------------|
| Water | 20 |
| Carbohydrates | |
| Sucrose | 35 |
| Glucose | 6 |
| Fructose | 9 |
| Other reducing sugars | 2 |
| Other carbohydrates | 3 |
| Nitrogenous compounds | |
| Crude protein | 4.5 |
| Amino acids (mainly aspartic and glutamic acids) | |
| -and some pyrrolidine carboxylic acid | 0.5 |
| Non-nitrogenous acids | |
| Organic acids (mainly aconitic acid) | 5 |
| Wax, sterols, gums, pigments and polysaccharides | 5 |
| Mineral salts or ash | |
| potash, lime, sulfates, magnesium salts, silica, chlorides, | |
| -phosphate, sodium, aluminum and iron oxides | 10 |
| Vitamins | |
| vitamins A, biotin, niacin, pyridoxine, pantothenic acid, | |
| -riboflavin and thiamine | Trace |

4. Crystallization The syrup is further evaporated in a vacuum pan until the sugar saturation point is reached. "Seed sugar" is added to serve as nuclei for sugar crystallization. Syrup is continuously added as the water evaporates and the sugar crystals grow. When the pan is full, the crystals and syrup form a dense mass called a "massecuite". The content of the pan is discharged into a centrifuge where raw sugar is separated from the mother liquor. The process is repeated as many times as practical, normally three.

5. Purging and reboiling massecuite The massecuite is centrifuged to separate the raw sugar from the mother liquor. The first mother liquor is the "A molasses". It is returned to the vacuum pan to be reboiled, producing a B sugar and a B molasses. A and B sugar are commercial output of the factory. B molasses is reboiled to yield C sugar and a final or blackstrap molasses. The C sugar is used as "seed grain" in the sugar crystallization process.

Generally, solid content in molasses is measured using a refractometer and is expressed as percent Brix. The degree Brix is defined as the percentage by weight of sucrose in a pure sugar solution (Meade, *et al.*, 1977). The Brix of an impure sugar solution is normally higher than the actual content solids obtained by drying because of the higher densities of nonsugar substances present in the solution. The sucrose is measured using a polarimeter. "Pol" values are the percent sucrose in the solution. Purity is Pol/Brix and is a measure of percent sucrose on solids.

Blackstrap molasses is used widely in many applications. Its major uses are as follows (Ruter, 1975):

1. Animal feed The feed value is based on the content of carbohydrates, minerals and trace elements. Molasses is sold as high or low sugar content on Brix measurement. It has been utilized as animal feed for more than 100 years in Europe and USA (Olbrich, 1970 and ITC, 1972). Molasses form (bulk, liquid, spray or dry forms) and concentration for feed applications are dependent on the type of animal. Because of the relatively low protein content in molasses, some proteins are added for the feeding of

monogastric animals that cannot synthesize the missing amino acids in molasses. In contrast, ruminants have bacterial flora in their rumens that can transform those inorganic nitrogenous compounds into readily utilized forms.

2. Desugarization of molasses It is considered to be one of the most important molasses uses. Sugar can be recovered in the form of sucrose, or as a sugar which contains both sucrose and invert sugar.

2.1 Recovery of sucrose: Sucrose can be separated from other substances by using semipermeable membrane (osmosis), precipitation by metals of calcium, strontium, barium or lead (Olbrich, 1970), or passing molasses through a strong acidic ion exchange resin, which is subsequently washed by water (Ruter, 1975).

2.2 Production of liquid sugar: Non-sugar substances can be removed by chemical and physical treatment of molasses to finally obtain clean sugar syrups. Sugar crystallization is not necessary. The processes used include filtration, ion exclusion, ion exchange, decolorization and adsorption. The purified liquid sugar is used widely in soft drinks, fruit juices, lemonades, cakes, sweets, etc.

3. Fermentation of molasses Many industrial plants use molasses as a substrate for microbial fermentations.

3.1 Yeast production: Baker's yeast (active dry yeast) is sold as pressed or dry yeast. It is used as a leavening agent and in bakeries. *Saccharomyces cerevisiae* is the organism of choice for Baker's yeast. Before yeast fermentation, molasses is clarified by applying heat and subsequently removing sediments. The clear solution is then fed to a fermenter. The molasses is supplemented with certain salts, such as ammonium phosphate, ammonium sulfate, ammonia or magnesium sulphate to meet the requirement of the organisms.

Food and feed yeasts (dry yeasts) are generally by-products from breweries. Yeast cells are not active due to the drying process. They are used as sources of proteins and vitamins to enrich food and animal feed (Peppler, 1967). They may be hydrolyzed by

hydrochloric acid and admixed into flavouring agent for seasonings. They provide nitrogen sources for the production of vitamins, nucleic acids and various enzymes. *Torulopsis utilis* and *Candida tropicalis* are commonly used organisms in feed yeast production.

3.2 Alcohol production: Ethyl alcohol fermentation is one of the oldest industrial uses for molasses. *Saccharomyces cerevisiae* is the organism used. Inverted sugar (normally glucose) is converted into ethyl alcohol and carbon dioxide. Some fusel oil, a mixture of higher boiling alcohols mainly amyl, isoamyl, isobutyl and normal propyl alcohol, is formed during fermentation, approximately 0.1-0.5% by volume to the ethanol volume. Vinasse is the fermentation residue from molasses after alcohol distillation. It can be used as fertilizer and glycerol production (Olbrich, 1970).

3.3 Organic acid production: There are many organic acids that are or can be produced by fermentation from molasses. Acetic acid, citric acid, glutamic acid, itaconic acid, aconitic acid, fumaric acid, malic acid and lactic acid are some examples.

MATERIALS AND METHODS

I. Organism and Maintenance

Lactobacillus casei subspecies *rhannosus* (ATCC 11443) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Stock cultures were maintained on *Lactobacillus* MRS agar plates (Difco, Detroit, MI), kept at 4°C and were subcultured biweekly. *Lactobacillus* MRS medium is composed of the following (values/liter).

| | |
|------------------------------|-------|
| Bacto proteose peptone No. 3 | 10 g |
| Bacto beef extract | 10 g |
| Bacto yeast extract | 5 g |
| Bacto dextrose | 20 g |
| Tween 80 | 1 g |
| Ammonium citrate | 2 g |
| Sodium acetate | 5 g |
| Magnesium sulfate | 0.1 g |
| Manganese sulfate | 0.05 |
| Dipotassium phosphate | 2 g |

The final pH of the medium was 6.5.

II. Analytical Methods

A. Cell density determination. Cell density was determined by measuring culture absorbance at 660 nm using a Gilford Response II spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH). The absorbance readings of turbid samples were corrected using the method of Toennies and Gallant (Toennies and Gallant, 1949).

B. Cell mass determination. Cell mass was determined from a standard curve of absorbance versus cell dry weight. The standard curve was created by growing cells in *Lactobacillus* MRS medium. The cells were harvested by centrifugation at 5,000 x g for 20 minutes and then were washed three times with distilled water. Cell pellets were resuspended

in 10 ml volumes of distilled water to various densities. The absorbance at 660 nm of these dilutions were measured then 5 ml of each dilution were transferred to aluminum weighing dishes and dried at 90°C to constant weight. Cell mass displayed a linear correlation with absorbance, with the following relationship:

$$\text{cell mass (mg/ml)} = \frac{A_{660} + 0.0014738}{4.501}$$

C. Determination of sugars

C.1 Reducing sugars. Glucose and fructose were determined by using the dinitrosalicylic acid (DNS) assay (Sumner, 1921). DNS reagent is composed of the following (values/ liter):

| | | |
|---------------------------|-------|----|
| 3,5-dinitrosalicylic acid | 7.49 | g |
| sodium metabisulfate | 5.86 | g |
| sodium hydroxide | 13.98 | g |
| sodium potassium tartrate | 216.1 | g |
| phenol | 5.37 | ml |
| Distilled water | 1000 | ml |

A sample, 0.5 ml, was mixed with 2 ml of DNS reagent. The solution was immersed in a boiling water bath for 5 minutes. The solution was cooled to room temperature prior to adding 5 ml of distilled water and then vortexed. The absorbance at 560 nm of the solution was read using a Gilford Response II spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH). The concentration of reducing sugars in the sample were calculated from a standard curve using glucose where:

$$\text{Reducing sugar (g/l)} = \frac{A_{560} + 0.00098}{0.64865}$$

C.2 HPLC sugar analysis. Sucrose, glucose and fructose were determined by High Performance Liquid Chromatography (HPLC) using an AminexR HPX-87K carbohydrate analysis column (300 x 7.8 mm; Bio-rad, CA). The injection volume of the sample was 20 ul. The column was eluted with 0.01M K₂SO₄ at a flow rate of 0.6 ml/min for 20 minutes.

D. Determination of L-(+) lactic acid

L-(+) lactic acid was determined using the enzyme L- (+) lactate dehydrogenase (cytochrome b; EC 1.1.2.3 (Sigma, St. Louis, MO)) (Appleby and Morton, 1959). Sodium pyrophosphate, 0.1 M at pH 8.4, was used as a buffer and 0.0083 M potassium ferricyanide provided Fe^{3+} for L- (+) LDH to convert lactic acid into pyruvic acid. In a 2.5 ml cuvette, 0.1 ml of 0.01 M EDTA, 0.6 ml of potassium ferricyanide solution and 1 ml of sodium pyrophosphate solution were mixed with 0.5 ml of sample. The enzyme, L- (+)LDH, was rapidly added and mixed with the reagents in the cuvette. The rate of initial reduction in absorbance at 420 nm was used as a measure of substrate concentration L- (+) lactic acid. The kinetics were monitored for 10 minutes using a Gilford Response II spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH). The amount of product in a sample was calculated from a standard curve where:

$$\text{L- (+) lactic acid (g/l)} = \frac{\text{Initial rate} + 8.800\text{e}^{-5}}{0.7416}$$

III. Growth

A. Shake flasks The bacteria were grown in an appropriate broth according to each experiment. The cultures were incubated in a shaking incubator (New Brunswick Scientific, Edison, NJ) at 40°C, except for the temperature optimization experiment. The agitation rate was 200 rpm. The inoculation cultures used for 1 liter fermentations were grown in Lactobacillus MRS broth.

B. 1 liter fermentors. L-(+) lactic acid was produced in batch fermentation using a BioFlo II fermentor (New Brunswick Scientific, Edison, NJ) or an Applikon fermentor (Applikon, Holland). Fermentations were inoculated at a 5% (v/v) inoculum with log phase cultures from shake flasks. The absorbance at 660 nm of the inocula were between 3-5. The pH of the fermentation was maintained at 5.5 (except for the experiments of culture pH effect), agitation rate was 200 rpm and temperature was 40°C. NaOH (10%) was used to maintain pH.

IV. Cell Immobilization and Bioreactors

A. Solid supports. Throughout the study, 8-20 mesh untreated granular activated carbon (Sigma, Detroit, MI) was used as the solid support. The only exception was in the solid support test experiment which used perlite, polyurethane foam or polystyrene foam.

B. Bench scale continuous bioreactor

B.1 Bench (10) ml bioreactor: *Lactobacillus casei* subsp. *rharnosus* was grown in 250 ml shake flasks containing 100 ml of broth culture. Upon reaching log phase, the 100 ml culture was transferred using a peristaltic pump, into a 10 ml reactor containing 3.4 g of 8-20 mesh sterilized granular activated carbon (Sigma, St. Louis, MO). The column was constructed from a jacketed 10 ml syringe. The jacket had ports to allow water to flow in and out for temperature control. The media feed was upflow. A break tube was added to the effluent line in order to prevent back contamination. The operation was set up as shown in Figure 13. After culture addition, the bioreactor was continuously flushed with a fresh media for 48 hours, to allow maximum cell growth and attachment at a flow rate of 10 ml/hr until the reactor reached a steady state.

B.2 Chemostat (400 ml) bioreactor: *L. casei* subsp. *rharnosus* was prepared for immobilization as above. The bacteria were transferred to a 400 ml BioFlo II chemostat fermentor (New Brunswick Scientific, Edison, NJ) which was filled with 100 g of untreated activated carbon. Temperature was controlled at 40°C. The fermentor was equipped with two internal media recycling lines that were connected to peristaltic pumps to allow media mixing in the vessel. The culture pH was controlled with 5% NaOH solution. The operational set-up is illustrated in Figure 14.

C. Pilot plant. A pilot column was constructed of plexiglass. The column was 7 x 25 x 6 inches. Inside the column were three boxes, each 5.5 x 5.5 x 4.5 inches. Two of the four sides of each box were made of mesh small enough to hold the solid supports and large enough to allow liquid to pass through. Spacing between each box was 1.7 inches. Each box contained 450 grams of granular activated carbon. On the top of the column were ports for

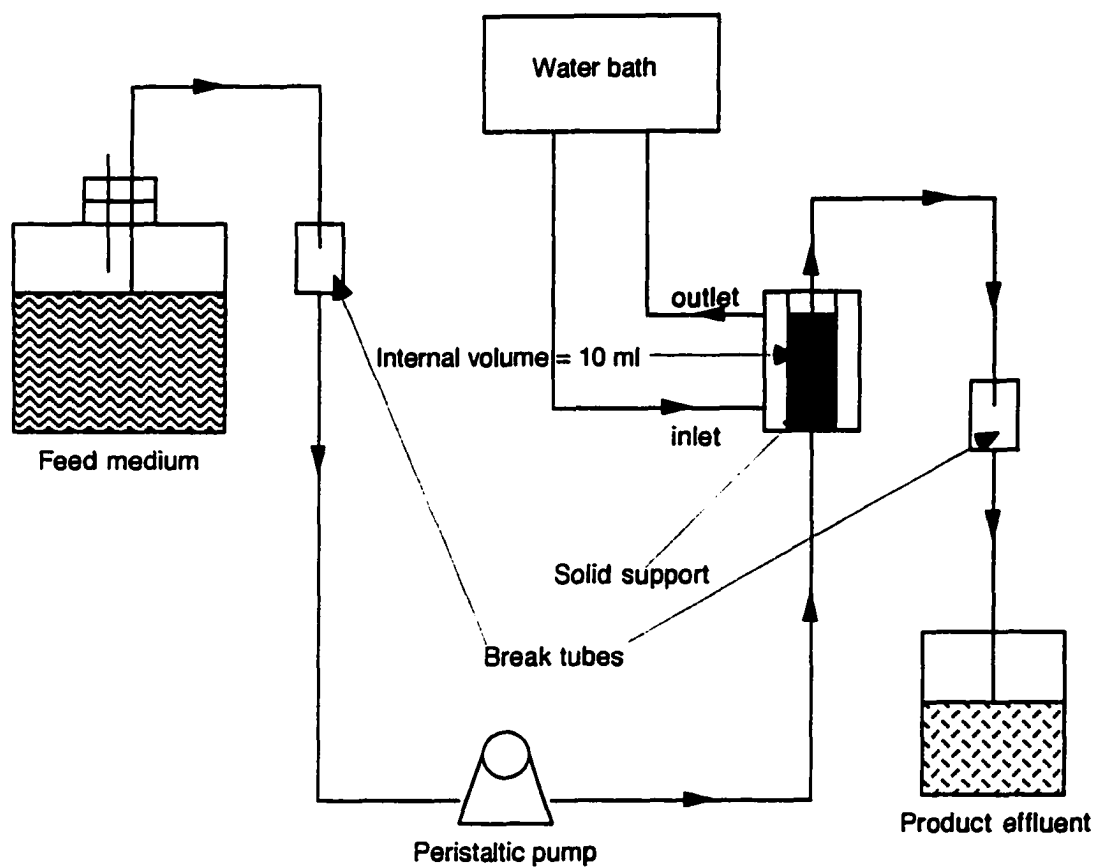


Figure 13. The bench-scale bioreactor. The column has a working volume of 10 ml. It contains 3 grams of granular activated carbon as cell support.

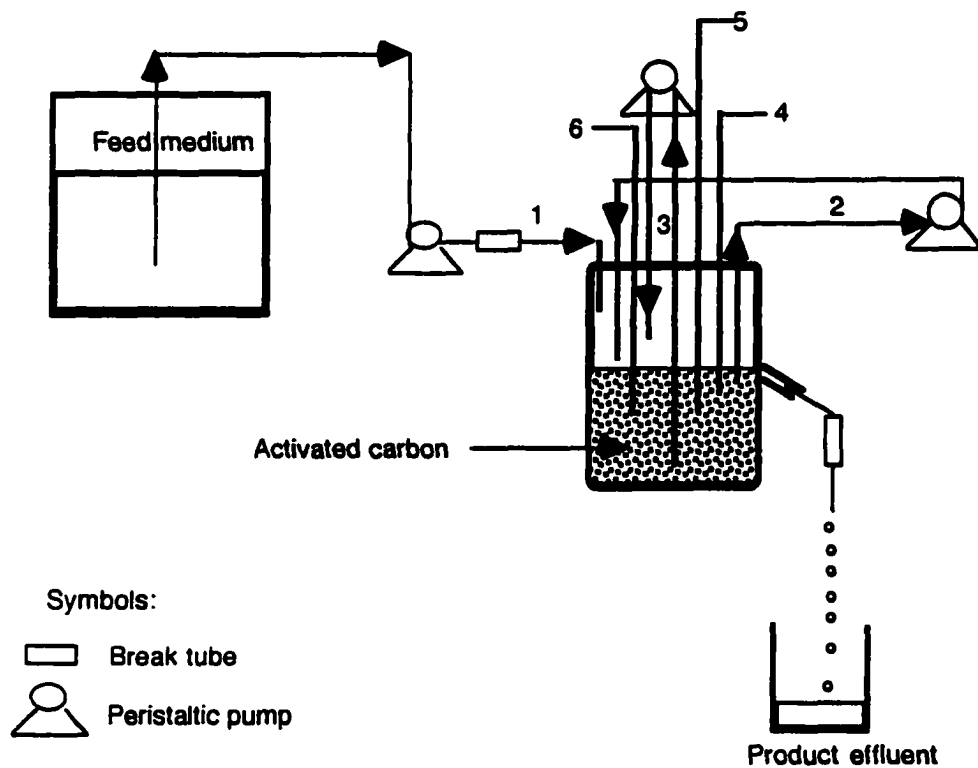


Figure 14. The operation set-up of a 400 ml immobilized cell fermentor. 1, Feed line; 2 and 3, Media recycling lines; 4, pH Probe, 5; Heating probe; 6, Temperature probe. Diagram is not to scale.

probes for pH and temperature as well as sample ports and fluid inlets for pH control. On each end of the column were inlet-outlet holes, each 3.5 inches from the bottom. The outlet was connected to a break tube. Media were fed to the bioreactor with a centrifugal pump. Five liters of clorox were used to sanitize the column prior to use. Two column volumes of sterile hot water were passed through the column before the feed of 10% sterile molasses (w/v) was started. Cultures were grown in Erlenmeyer flasks containing 500 ml of broth. When they were in log phase, cultures were transferred to the aseptic pilot column containing 5.36 liters medium. After inoculation, the bioreactor was allowed to incubate for 48 hours to allow the cells to immobilize onto the solid supports prior to starting the feed. The temperature of the column was controlled at 42°C with a water bath. Feed was prepared in 20 liter carboys that were sterilized by autoclaving. The operational set-up is shown in Figure 15.

V. Optimization of Fermentations

A. Temperature: For batch fermentation, cells were grown in media containing Lactobacillus MRS media or molasses, 8% Brix. Media were adjusted to pH 5.5 prior to inoculation. The experiments were conducted as 100 ml in 250 ml Erlenmeyer flasks at 28, 33, 37, 40, 42, 45 and 50°C. The agitation rate was 200 rpm. Samples were analyzed for L- (+) lactic acid, pH and cell growth.

For immobilized cell fermentations, four liters of molasses, 8% Brix, was adjusted to pH 5.5 and was autoclaved at 121 psi for 45 minutes. The medium was fed to the 10 ml bioreactor at a constant flow rate of 11.4 ml/hr. For each temperature, the fermentation was run for 3 days to achieve steady state. L- (+) lactic acid was analyzed at various times.

B. Initial pH: Batch cultures were grown in 100 ml broth in 250 ml Erlenmeyer flasks at 200 rpm agitation rate, using Lactobacillus MRS media or molasses, 8% Brix. Temperature was fixed at 40°C. Media pH's were adjusted initially using 40% NaOH and concentrated HCl. Samples were collected for growth, L-(+) lactic acid and pH drop analyzed over time. For pH control experiments, molasses (8% Brix) were supplemented with 0.4% yeast extract and were adjusted to pH 4, 5, 5.5 and 7 prior to autoclaving. The culture

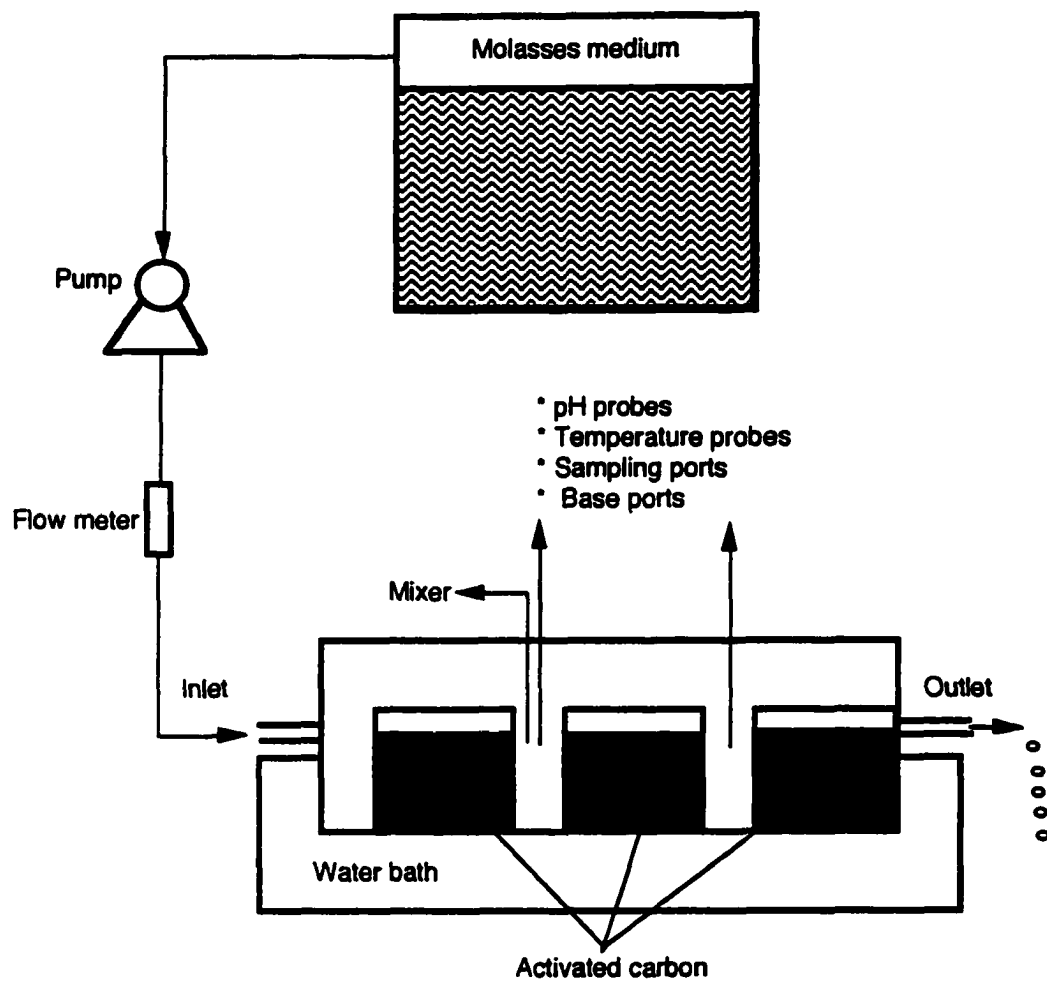


Figure 15. Pilot plant. The unit has a working volume of 5.36 liters. The diagram is not to scale.

fermentations were controlled to pH 4, 5, 5.5 and 7. Samples were analyzed for L- (+) lactic acid and residual sugar.

For immobilized cell fermentations, blackstrap molasses were prepared at various pH's, but at a constant solids concentration of 8% Brix. Media were sterilized by autoclaving. Tests were conducted at two feed flow rates (2.2 and 4.4 ml/hr). Each condition was monitored for 60 hours. Before switching media, the bioreactor was rinsed with 10 volumes of sterile 0.8% NaCl. Samples were collected for lactic acid analysis, cell count and pH measurement.

C. Sodium azide: Sodium azide was added into Lactobacillus MRS broth or molasses, 8% Brix, at 0, 0.8, 1.54, 7.69, 15, 30 and 60 mM to see if this compound can be used to prevent contamination of *L. casei* subspecies *rhamnosus* column. The media was adjusted to pH 5.5 prior to autoclaving. The seed cultures were grown in Lactobacillus MRS broth and the tested media were inoculated to after they reached 3-5 absorbance at 660 nm. Batch fermentations were conducted at 40°C and 200 rpm agitation rate in 250 ml Erlenmeyer flasks containing 100 ml media. Absorbance at 660 nm and pH drops were monitored. Cell concentrations were determined from absorbance measurements. The K_i determination of sodium azide on *L. casei* subsp. *rhamnosus* growth was adapted from the function for a noncompetitive inhibitor to an enzyme (Aiba, *et al.*, 1973). The K_i is the concentration of an inhibitor that inhibits 50% cell growth.

For immobilized cell fermentation, sodium azide (0, 0.01 and 0.05%) was added into blackstrap molasses (8% Brix) and adjusted to pH 5.5 prior to autoclaving. The media were fed to the bioreactor at a constant flow rate of 2.2 ml/hr. Samples were analyzed for L- (+) lactic acid.

D. Flow rates: Blackstrap molasses was diluted to 8% Brix with water. The media pH was adjusted to pH 5.5. The medium was fed to the column at flow rates of 2.2, 4.4, 7.6, 11.4, 18, 24 and 31 ml/hr by using a peristaltic pump. For each flow rate, the fermentation was monitored for 60 hours.

E. Extracellular lactic acid: Batch cultures were grown in 100 ml broth in 250 ml Erlenmeyer flasks. Cultures were grown in *Lactobacillus* MRS media, or molasses, 8% Brix, which were supplemented with 0, 4, 10, 240, 360, 440 or 610 mM lactic acid. The agitation rate was 200 rpm. Temperature was fixed at 40°C. Initial media pH was adjusted to 5.5. Samples were analyzed with time. For immobilized cell fermentation, lactic acid (0, 59.9, 139.9 or 222 mM) was added into blackstrap molasses (8% Brix). The media were adjusted to a final pH of 5.5. The media were fed at 11.4 ml/hr flow rate. Effluent L- (+) lactic acid was determined. The K_i for lactic acid inhibition is determined the same as for the sodium azide inhibition.

F. Effect of substrate concentration on lactic acid production. Batch experiments were conducted on 100 ml cultures in 250 ml Erlenmeyer flasks containing *Lactobacillus* MRS broth or molasses without pH control, and as 1.0 liter fermentations with pH control, at pH 5.5 (description in section III.B). Glucose concentrations in *Lactobacillus* MRS media were adjusted to a desired concentration. Molasses media were made to desired concentrations by diluting Louisiana blackstrap molasses in water. For molasses concentration greater than 20% (w/v), the pH had to be adjusted approximately 1 pH unit above the desired to counteract the drop observed on autoclaving. Fermentation conditions were 40°C and 200 rpm agitation rate. The inocula were prepared on *Lactobacillus* MRS broth, with absorbance at 660 nm between 3-5 used for inoculation, 5%. Five milliliter samples were collected for analysis with time.

For continuous, immobilized cell fermentations, different molasses concentration media were adjusted to pH 5.5 and fed to the bioreactor at flowrates of 2.2 and 4.4 ml/hr. Product effluent was analyzed for L- (+) lactic acid, pH and sugars.

For the pilot plant, molasses was prepared to desired concentrations and pH's, in 20 liter carboys, and sodium azide was added (0.005% w/v), to minimize contamination that develops inside the column after extended operation, prior to autoclaving. The medium was

fed at the desired flow rates, 60, 300 and 600 ml/hr. Samples were collected at various parts across the column and from the effluent. Temperature was 40°C.

G. Effect of nutrient supplemented molasses on lactic acid production and cell growth: Cultures, 100 ml, were grown in molasses, 8% Brix, in 250 ml Erlenmeyer flasks, without pH control. Nutrient supplements tested were 0, 0.2, 0.4 and 0.8% (w/v) yeast extract. These media were adjusted to pH 5.5 prior to use. The inocula grown on Lactobacillus MRS broth culture, and had absorbances at 660 nm of 3-5 at less than 48 hours old. Samples were taken with time and analyzed for L- (+) lactic acid.

pH Controlled cultures were grown in 1 liter fermentors. Yeast extract, 0, 0.2, 0.4 or 0.8% (w/v) was added to 8% brix molasses before autoclaving. Fermentation conditions were 200 rpm and 40°C. The inocula were grown on Lactobacillus MRS broth for 48 hours as previously described. Samples were analyzed for growth and L- (+) lactic acid.

For immobilized cell fermentations, blackstrap molasses (8% Brix) with nutrient supplement (yeast extract, ammonium phosphate (dibasic), corn steep liquor or invertase) were used as feed. The media were adjusted to pH 5.5 prior to autoclaving. The media were passed through the 10 ml bioreactor at a constant flowrate of 11.4 ml/hr. Samples were analyzed with time for L- (+) lactic acid, pH, cell count and sugar utilization.

H. Effect of molasses inversion on lactic acid fermentation: Molasses, 8% Brix, were inverted by the addition of invertase, which was obtained from Baker's yeast (Sigma, St.Louis, MO). To 1 liter of 8% Brix molasses, 0, 2, 5 and 10 g invertase was added. The mixture was held at 56°C for at least 20 minutes before autoclaving. Molasses was also inverted by reducing pH to below 3.0 before heating for 4 hours. The acid invert molasses was adjusted to pH 5.5 prior to use. All molasses media were adjusted to pH 5.5 and were autoclaved prior to use. Fermentations were conducted in 1 liter fermentors, at 40°C and 200 rpm agitation rate, without aeration. NaOH (10%) was used to control pH.

For continuous fermentations, inverted molasses, pH 5.5, were fed to the bioreactor at a flow rate of 2.2, 4.4 and 11.4 ml/hr. Product effluent was analyzed for L- (+) lactic acid and fermentable sugar in molasses.

I. Effect of molasses clarification on lactic acid production. Molasses was clarified by heating above 95°C for 10 minutes and then allowing to cool. The clarified molasses was decanted and then was adjusted to 8% Brix, pH 5.5 prior to autoclave. Fermentations were conducted in 1 liter fermentors, at 40°C and 200 rpm agitation rate, without aeration. NaOH (10%) was used to control pH. For continuous fermentations, clarified molasses (pH 5.5) was fed at a flowrate of 11.4 ml/hr. Product effluent was analyzed for L- (+) lactic acid and fermentable sugar in molasses.

J. Effect of solid supports on cell immobilization and lactic acid production. Media, pH 6, containing 3% sucrose, 0.5% tryptone and 0.5% yeast extract were fed to a 10 ml bioreactor at the flow rate of 6 ml/hr. The fermentations were run for 5 days. Solid supports tested were activated carbon, perlite, polystyrene foam and polyurethane foam. All solid supports was sterilized by autoclaving prior to use. For calcium alginate bead preparation, sodium alginate, 4% (w/v), was sterilized and cooled before addition to *L. casei* subsp. *rhamnosus* culture in a ratio of 1:1. The culture was then pumped using a peristaltic pump through a 16 mm internal diameter tubing to a solution of 0.2 M CaCl₂. The beads were transferred to a 10 ml syringe column. Immobilized cells on each solid support, except for alginate beads, were monitored by electron microscopy.

VI. Adsorption of Lactic Acid by Activated Carbon

L- (+) lactic acid solution was adjusted to pH 4.0, 5.0 or 6.0 prior to autoclaving. After pH adjustment and sterilization, the solution had lactic acid concentrations of 4.5, 4.4 and 4.6 g/l for pH 4, 5 and 6 solution, respectively. Sterile lactic acid solution, 2 ml, was transferred into screw cap tubes containing 1.0 gram sterile activated carbon. The screw cap tubes were wrapped with parafilm before incubating at 40°C for 24 hours. L- (+) lactic acid concentration was analyzed before adding to activated carbon and after incubation. Residual lactic acid after

incubation was subtracted from the starting lactic acid concentration to determine lactic acid adsorption. This experiment was done in duplicate.

VII. Purification of Lactic Acid

Fermentation broth was centrifuged at 5,000 g for 30 minutes at 4°C to remove cell mass. The supernatant was passed through a basic anion exchange column containing 6.0 g of Amberlite IRA-400 (Sigma, St.Louis, MO). Prior to use, the resins were rinsed with 5% NaOH, in order to replace Cl group on the resins with OH group. Afterwards, the column was flushed with distilled water until the effluent pH was 8 or above. Spent broth, pH 8, was then passed through the resin packed column at a speed of 15 ml/min by a peristaltic pump. Subsequently, the column was rinsed with approximately 4 bed volumes of distilled water before the bound product was eluted with 2N HCl, 4 ml collecting fraction. The eluent was collected as a 4 ml fraction. The resins were recycled by rinsing with 5% NaOH and distilled water until the effluent pH was 8. Purification efficiency was determined as percent recovery of lactic acid.

VIII. Scanning Electron Microscopy (SEM)

Solid support particles with attached biofilm were removed from the bioreactor and immediately fixed for scanning electron microscopy (SEM) in equal parts of growth medium and 4% (v/v) glutaraldehyde for 24 hours. They were rinsed in 3 changes of 0.1 M sodium cacodylate buffer at pH 7.1 for at least 1 hour prior to be post-fixed in 2% OsO₄ in 0.05 M sodium cacodylate buffer for 1 hour. Samples were dehydrated in an ethanol wash series, then critical point dried in a Denton DCP-1, attached to specimen mounts, sputter coated with gold/palladium in an Edwards S-150, and examined and photographed in a Cambridge 280 Stereoscan SEM.

IX. Calculated Parameters

1. Dilution rate (hr^{-1}), D: The dilution rate is the function of a flow rate to a working volume of the reactor.

$$D = \frac{\text{Flow rate (l/hr)}}{\text{Working volume (l)}}$$

2. Volumetric productivity (g/l-hr): For batch fermentation, productivity is a function of lactic acid produced to duration time of fermentation while for continuous fermentation is the function of the dilution rate and lactic acid produced.

$$\text{Batch (g/l-hr);} \quad = \quad \frac{\text{Lactic acid produced (g/l)}}{\text{Fermentation time (hr)}}$$

$$\text{Continuous (g/l-hr)} \quad = \quad \text{Lactic acid produced (g/l)} \times D$$

3. Utilized substrate: The residual substrate in a culture broth is subtracted from the initial substrate concentration to yield the utilized substrate.

4. Yield (%): Yield is the ratio of lactic acid produced to utilized substrate, multiplied by 100.

5. Molar conversion (moles lactic acid produced/moles glucose or hexose utilized):
Molar conversion is a ratio of lactic acid produced (mole/l) to glucose or hexose (mole/l).

RESULTS

I. L- (+) Lactic Acid Production

A. Shake flask studies

A.1 Growth and production on *Lactobacillus* MRS media

Lactic acid production in the synthetic media (MRS) was used as the baseline for comparison of molasses based fermentations. *Lactobacillus casei* subspecies *ramnosus* utilized glucose and produced L- (+) lactic acid when grown on *Lactobacillus* MRS media. This media contains 24 g/l glucose with other nutrients. There was a pH drop of 2 units (from pH 5.5 to 3.5). Without pH control, the bacteria utilized only 20 g/l of the glucose (85%) (Fig. 16). L- (+) lactic acid in the media increased in parallel with cell mass. The yield of the fermentation was 19 g/l L- (+) lactic acid, a 1.9 molar conversion of the glucose utilized to lactic acid produced.

L- (+) lactic acid production from *L. casei* subsp. *ramnosus* at seven different temperatures, 28, 33, 37, 40, 42, 45, and 50°C, was monitored in *Lactobacillus* MRS media. *L. casei* subsp. *ramnosus* did not grow at 50°C, and showed an extended lag phase when grown at temperatures on either side of 40°C (Fig.17). At 40°C, the bacterium produced the most L- (+) lactic acid (19 g/l) and showed a 1.9 molar conversion of the glucose (Fig. 18). Decreasing the fermentation temperature to 37, 33 or 28°C, 14.8, 12.2 and 11.6 g/l of L-(+) lactic acid were produced, respectively. When the fermentation temperature increased from 40°C to 42, 45 or 50°C, L- (+) lactic acid production decreased to 15.1, 14.8 and 0 g/l, respectively. Optimum temperature for lactic acid production of *L. casei* subsp. *ramnosus* was 40±2°C.

Culture pH dropped directly with lactic acid production. When the starting pH was 5.5, the lag phase was shortest compared to other starting pH values (Fig. 19). At an initial pH between pH 3 and 3.5, cells did not grow. When the media pH was 4, cells grew, but more slowly than at higher pH values. The molar conversion of the glucose to L- (+) lactic acid was

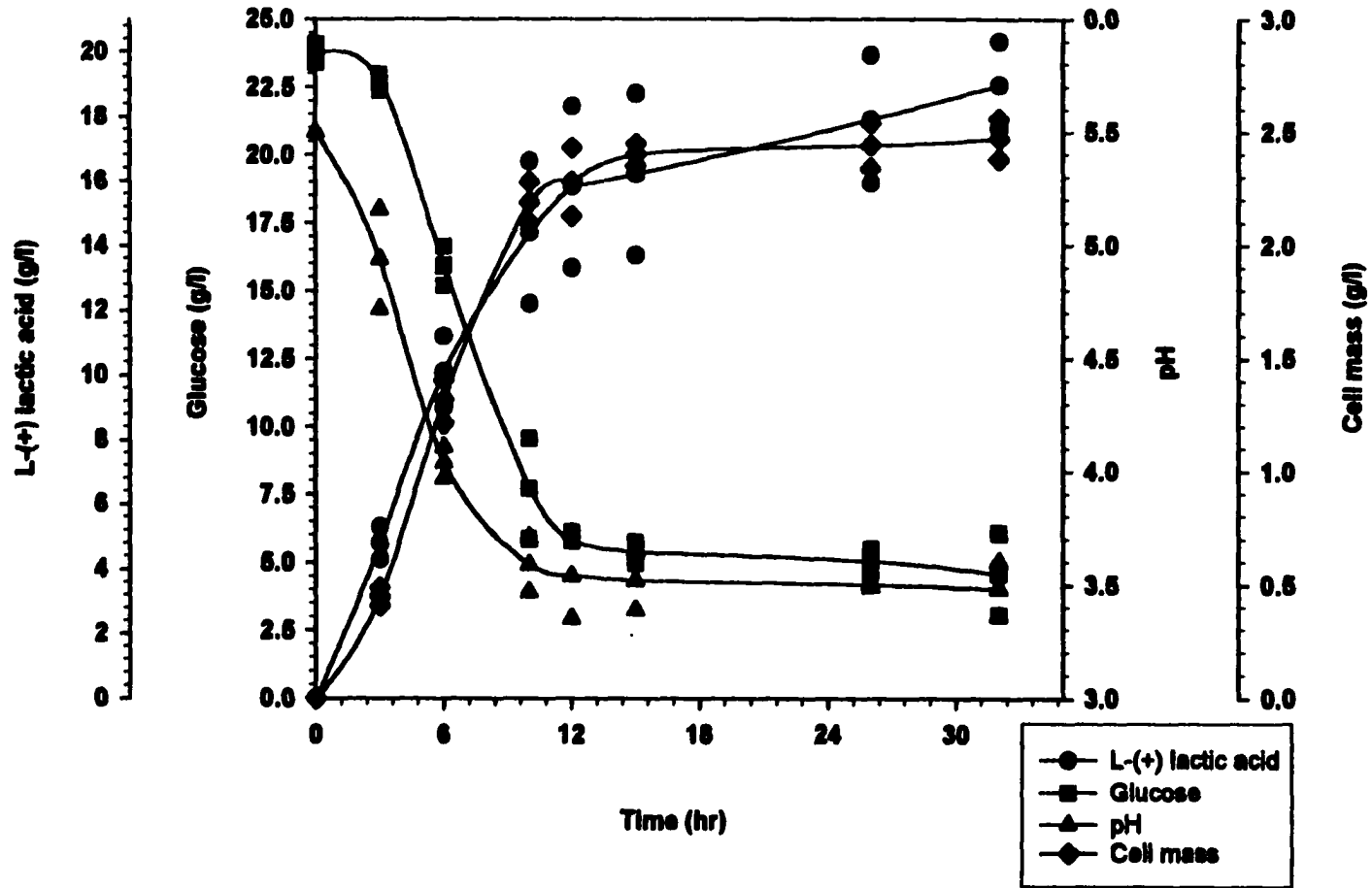


Figure 15. Growth and L-(+) lactic acid production by *L. casei* subsp. *rhamnosus* in Lactobacillus MRS media. These experiments were conducted as 100 ml cultures in 250 ml shake flasks at 40°C, 200 rpm agitation rate. The starting glucose concentration was 24 g/l. The initial pH was 5.5. The experiments were done duplicately. The range bar for each data point show the lowest and highest points.

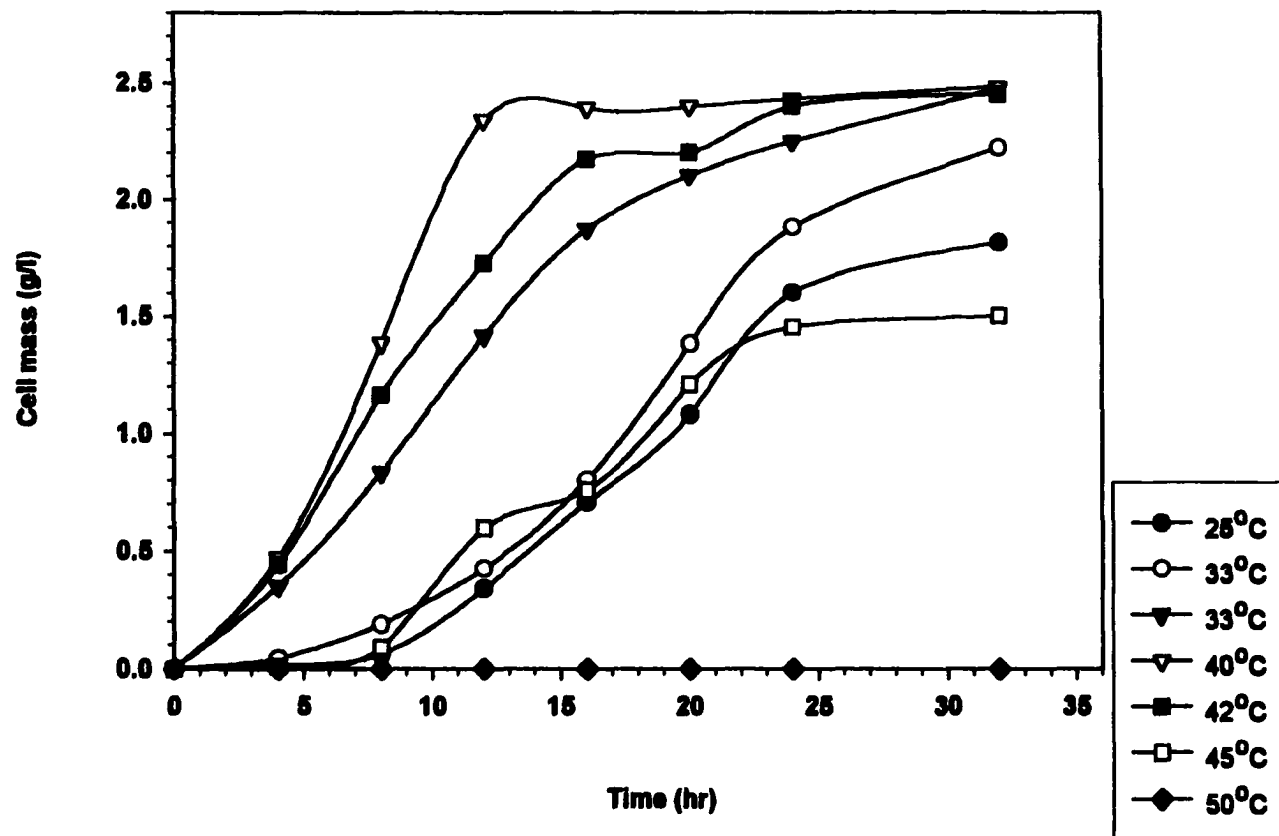


Figure 17. Growth of *L. casei* subsp. *rhamnosus* versus time at various temperatures, 28, 33, 37, 40, 42, 45 and 50°C. These experiments were conducted as 100 ml cultures in 250 ml Erlenmeyer flasks at 200 rpm agitation rate. The *Lactobacillus* MRS media were adjusted to pH 5.5 prior to use. The initial glucose concentration was 24 g/l. The data points are the average values of duplicated experiments.

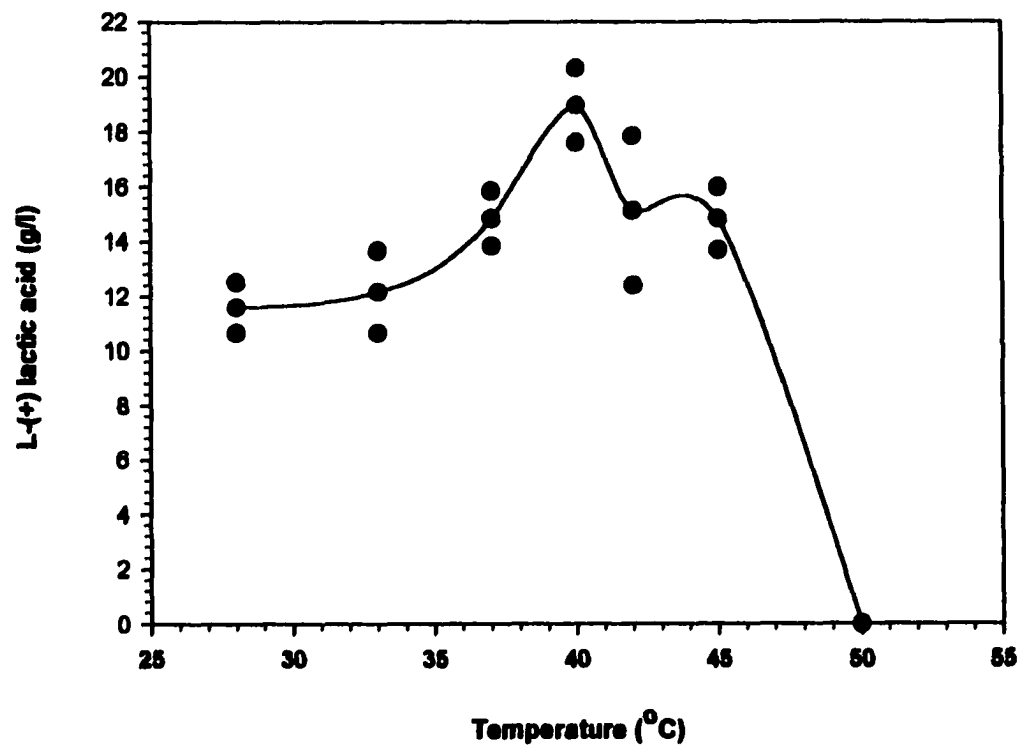


Figure 18. Effect of temperature on L-(+) lactic acid production by *L. casei* subsp. *rhamnosus*. These experiments were conducted as 100 ml cultures in 250 ml shake flasks. The starting media, 24 g/l glucose, pH was 5.5. The agitation rate was 200 rpm. The range bars show the lowest and highest values of lactic acid produced from duplicated experiments for each temperature.

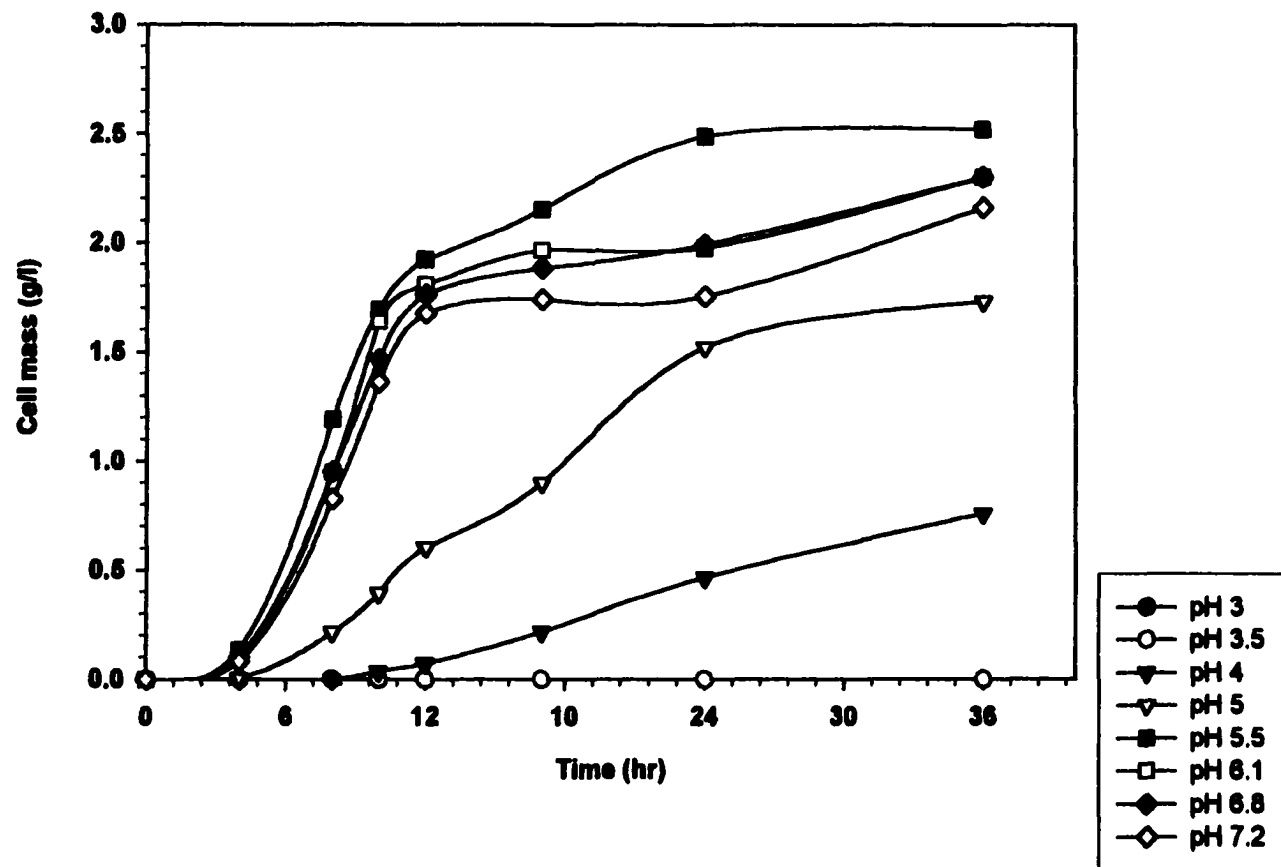


Figure 19. Growth of *L. casei* subsp. *rhamnosus* with time with different initial pH values. These experiments were conducted as 100 ml cultures in 250 ml shake flasks, at 40°C and 200 rpm agitation rate. The starting glucose concentration in the *Lactobacillus* MRS media was 24 g/l. The data points are the average values from duplicated experiments.

highest at pH 5.5 (1.9 mole L- (+) lactic acid /moles glucose) with 19.0 g/l lactic acid produced. Changing media pH above pH 5.5 did not change lactic acid production (Fig. 20).

Concentrations of 5, 10, 24, 42, 56, 81, 92, 111, 132 and 150 g/l glucose were tested to determine the effect of substrate concentration on L- (+) lactic acid production and cell growth in *Lactobacillus* MRS media. At a glucose concentration of 42 g/l, the conversion of glucose to lactic acid was 1.97 mole L- (+) lactic acid/mole glucose utilized. The L- (+) lactic acid production was 19.0 g/l. At 24 g/l glucose, 19 g/l of lactic acid was produced with a conversion of 1.91 mole lactic acid/ mole glucose. At low glucose concentration, 5 and 10 g/l, the molar conversion were 1.75 and 1.92 mole L- (+) lactic acid/moles glucose, with 4.0 and 8.6 g/l lactic acid produced, respectively. At glucose concentrations of 56, 81, 92, 111, 132 and 150 g/l, the amount of L- (+) lactic acid produced decreased to 18.6, 18.5, 17.6, 17.3, 17.2 and 17.0 g/l, respectively (Fig. 21). The results indicated that high concentration of substrate affected the glucose conversion to lactic acid due to the high osmolarity.

In order to quantitate the inhibitory effect of L- (+) lactic acid on cell growth, L- (+) lactic acid (0, 4.2, 7, 12.3, 20.3 g/l or 0, 0.04, 0.08, 0.13 and 0.22 M) was added to *Lactobacillus* MRS media prior to autoclaving. The media was adjusted to pH 5.5 prior to inoculation. *L. casei* subsp. *rhamnosus* growth was monitored for 48 hours and growth inhibition determined. L- (+) lactic acid gave a growth inhibition constant (Ki) of 10.8 g/l or 0.12 M (Fig. 22). The growth inhibition constant was determined at 50% cell growth inhibition.

For continuous lactic acid production (see section pilot plant) in a system which will operate continuously, for long periods of time, some form of contamination control is necessary. Sodium azide was chosen to control the aerobes which aggressively contaminated our pilot plant after a few weeks of operation. Sodium azide inhibits the functioning of cytochrome oxidases in aerobes and was not expected to affect lactobacilli. Sodium azide was added to *Lactobacillus* MRS media at concentrations of 0, 0.8, 1.5 and 7.7 mM prior to autoclaving. Sodium azide inhibited growth at both low and high concentrations. The inhibition constant (Ki) of sodium azide to *L. casei* subsp. *rhamnosus* growth was 2.1 mM

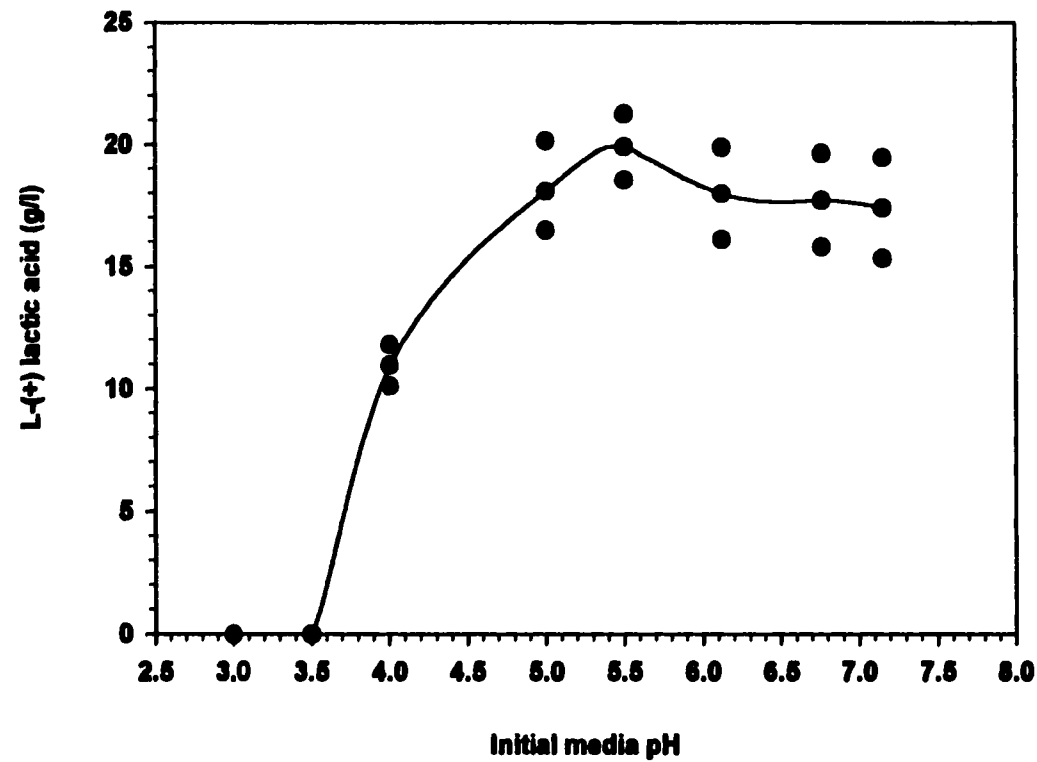


Figure 20. L-(+) lactic acid production by *L. casei* subsp. *rhamnosus* at different initial pH values. These experiments were conducted in Lactobacillus MRS media as 100 ml cultures in 250 ml shake flasks. Temperature was 40°C. The agitation rate was 200 rpm. The starting glucose concentration in the media was 24 g/l. L-(+) lactic acid determination was made when the cultures reached stationary phase (36 hours). The range bars show the lowest and highest values of duplicated experiments.

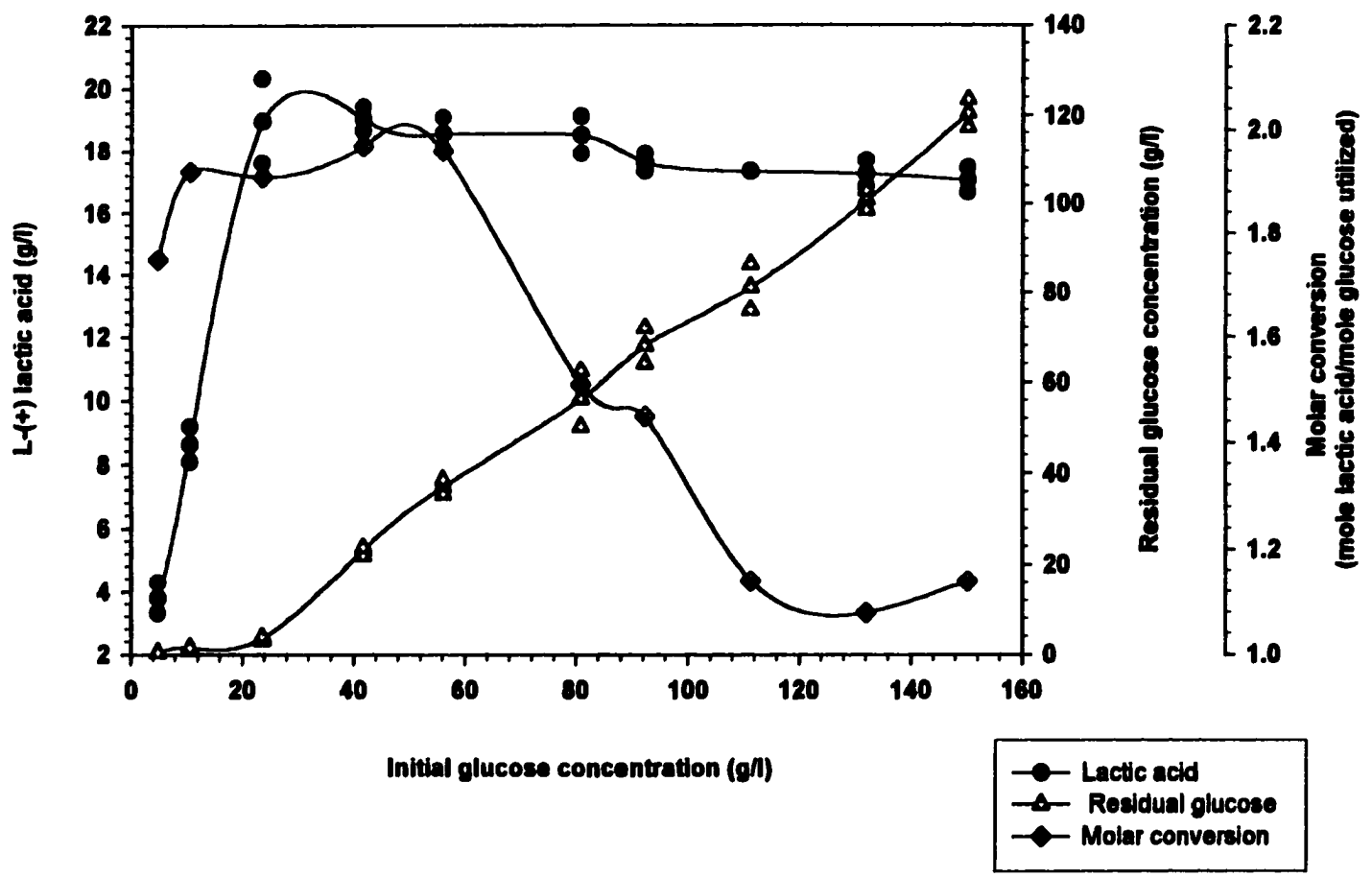


Figure 21 . Effect of initial glucose on lactic acid production by *L. casei* subsp. *rhamnosus* in MRS media. These experiments were done as 100 ml cultures in 250 ml Erlenmeyer flasks. Temperature was 40°C The agitation rate was 200 rpm. The starting media pH was 5.5. The cultures were grown for 24 hours before lactic acid was analyzed. The range bars show the lowest and highest values of duplicated experiments.

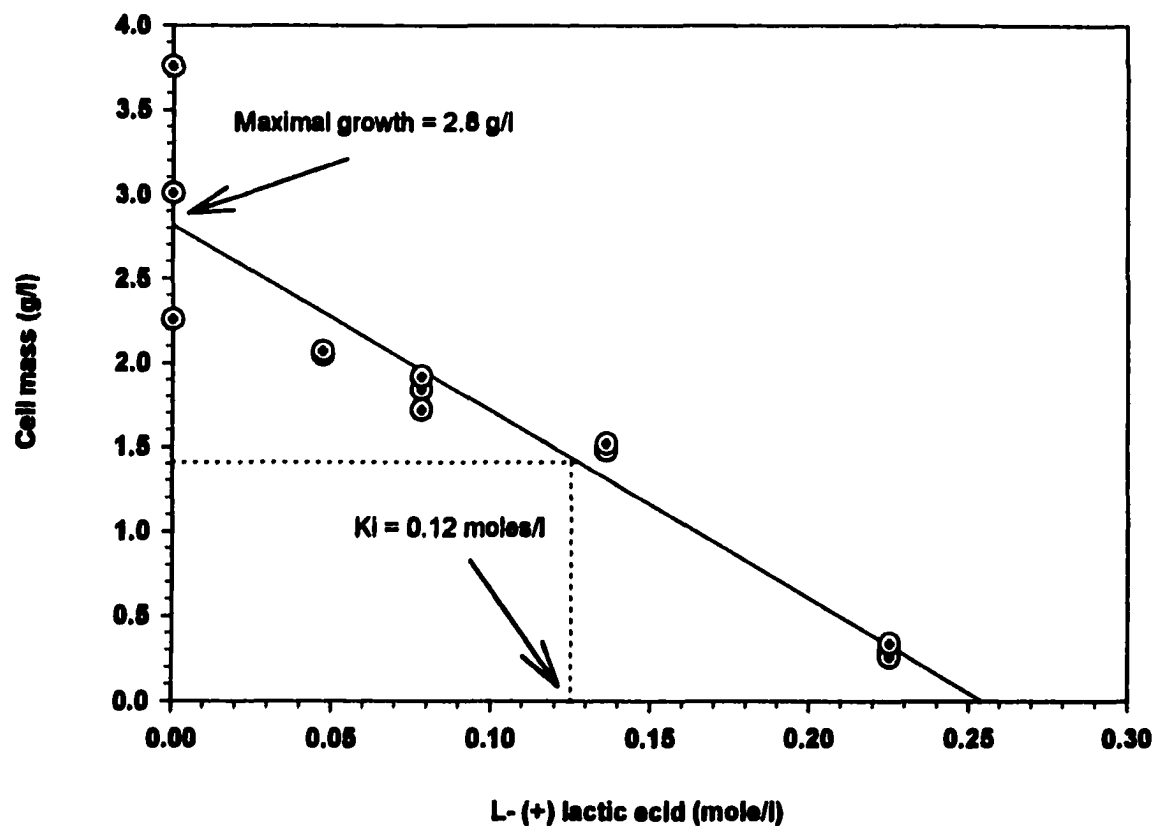


Figure 22. The inhibitory effect of external L-(+) lactic acid on *L. casei* subsp. *rhamnosus* growth. These experiments were conducted as 100 ml cultures in 250 ml shake flasks, 40°C and 200 rpm agitation rate. The *Lactobacillus* MRS media were supplemented with 0, 0.05, 0.08, 0.13 or 0.22 moles/l L-(+) lactic acid. The starting glucose in the media was 24 g/l. The initial pH was 5.5. The range bars show the lowest and highest values of duplicated experiments.

(Fig. 23). To examine inhibition by sodium azide on both *L. casei* subsp. *rharnosus* and normal contaminants, Lactobacillus MRS media were not sterilized but added 0, 0.15, 0.8 or 1.5 mM sodium azide were added. The inhibition constant (K_i) was not determined as a biphasic relationship with growth was found (Fig. 24).

A.2 Growth and production on blackstrap molasses

L. casei subsp. *rharnosus* grew on blackstrap molasses, but not as well as on MRS media. The molasses was diluted to 8% Brix (Brix = % solids), pH 5.5. Bacterial growth and lactic acid production was tested at seven temperatures. Growth of *L. casei* subsp. *rharnosus* on molasses responded to temperature in the same manner as on Lactobacillus MRS media. There was an extended lag phase at temperatures above or below 40°C (Fig. 25). At 40°C, the bacteria produced the most L- (+) lactic acid (16 g/l). At a temperature of 42°C, *L. casei* subsp. *rharnosus* grew more slowly and produced less L- (+) lactic acid, 9 g/l. When temperature increased to 45°C, the bacteria grew even more slowly and produced less L-(+) lactic acid, 5 g/l. At 50°C, *L. casei* subsp. *rharnosus* did not grow. At lower temperatures, 28, 33 or 37°C, the bacteria produced 9.1, 11.0 and 12.0 g/l L- (+) lactic acid, respectively (Fig. 26).

Molasses media was adjusted to different pH values prior to inoculation with *L. casei* subsp. *rharnosus*. At a starting pH of 3.2, the bacteria did not grow. When the starting pH was 4, the bacteria exhibited a prolonged lag phase and did not grow as well as at higher values (Fig. 27). The production at pH 4 was 1.2 g/l. Increasing pH to 5, the production increased to 8.2 g/l. Increasing pH to pH 5.5, the bacteria grew more rapidly and produced more L-(+) lactic acid (16 g/l). Increasing media pH to 6.2, 6.6, 6.8 and 7.6 did not improve production values, which were 8.2, 6.4, 4.5, and 2.7 g/l, respectively (Fig. 28).

Growth and L- (+) lactic acid production in molasses without additional nutrient supplementation were monitored for five different molasses concentrations, 1.8, 4.0, 7.3, 10.6, and 13.7% Brix. The experiments were run until growth ceased (36 hours). The amount of L- (+) lactic acid produced was dependent on substrate concentration to the point where

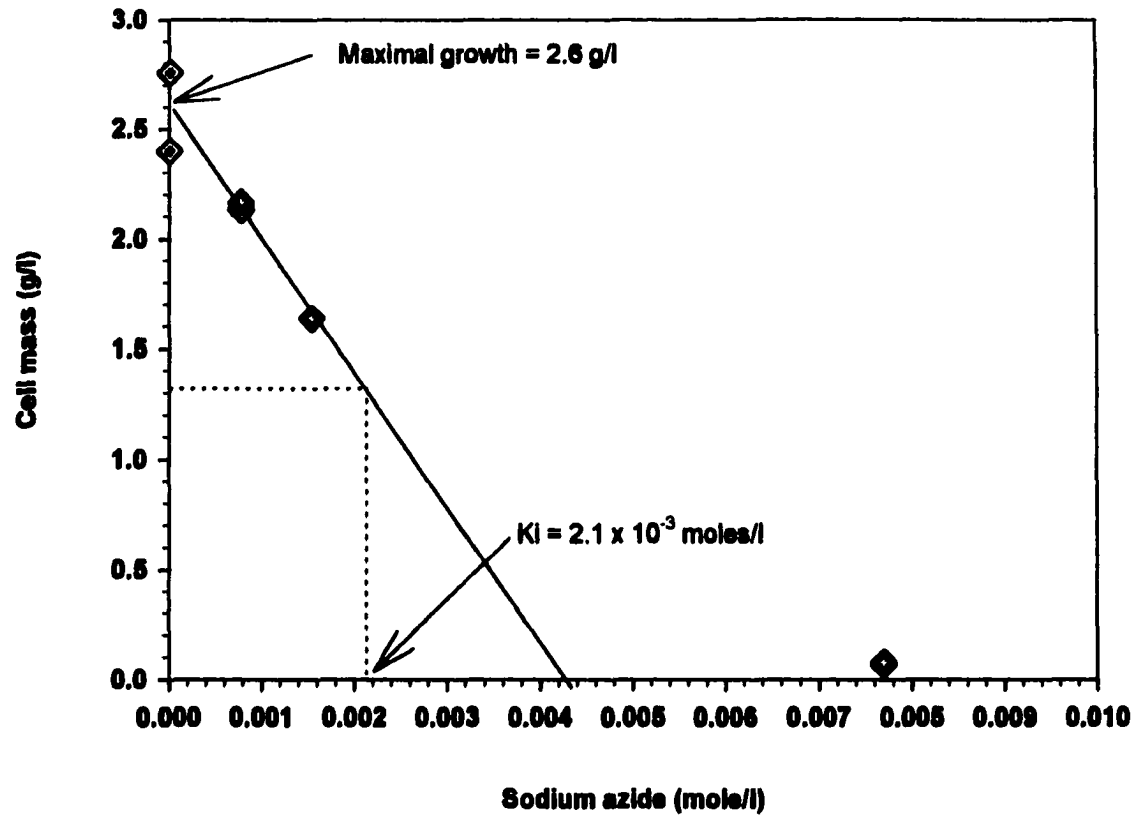


Figure 23. The inhibition of *L. casei* subsp. *rhamnosus* growth by sodium azide. These experiments were done dublicately in Erlenmeyer flasks at 200 rpm agitation rate, 40°C, without pH control. Lactobacillus MRS media was supplemented with 0, 7.69×10^{-4} , 1.54×10^{-3} or 7.69×10^{-3} moles/l sodium azide and the starting pH was 5.5. The determination of K_i was adapted from the method for determining the inhibition of a noncompetitive inhibitor to an enzyme (Alba, et al., 1973).

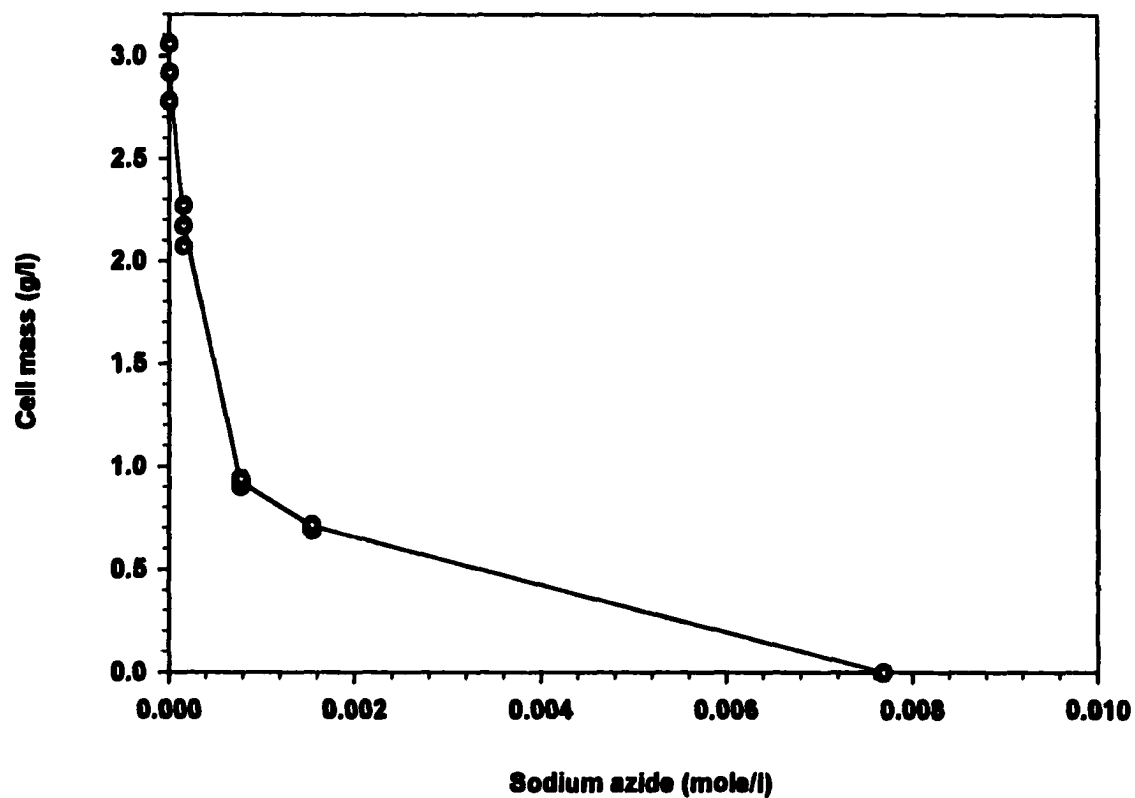


Figure 24. The inhibition of growth of microorganisms found during experiments, including *L. casei* subsp. *rhamnosus*, by sodium azide. These experiments were conducted as 100 ml culture in 250 ml shake flasks. The *Lactobacillus* MRS media was supplemented with 0, 1.54×10^{-4} , 7.69×10^{-4} , 1.54×10^{-3} or 7.69×10^{-3} moles/l sodium azide. The initial pH was 5.5. Temperature was 40°C. The range bars show the lowest and highest values of duplicated experiments.

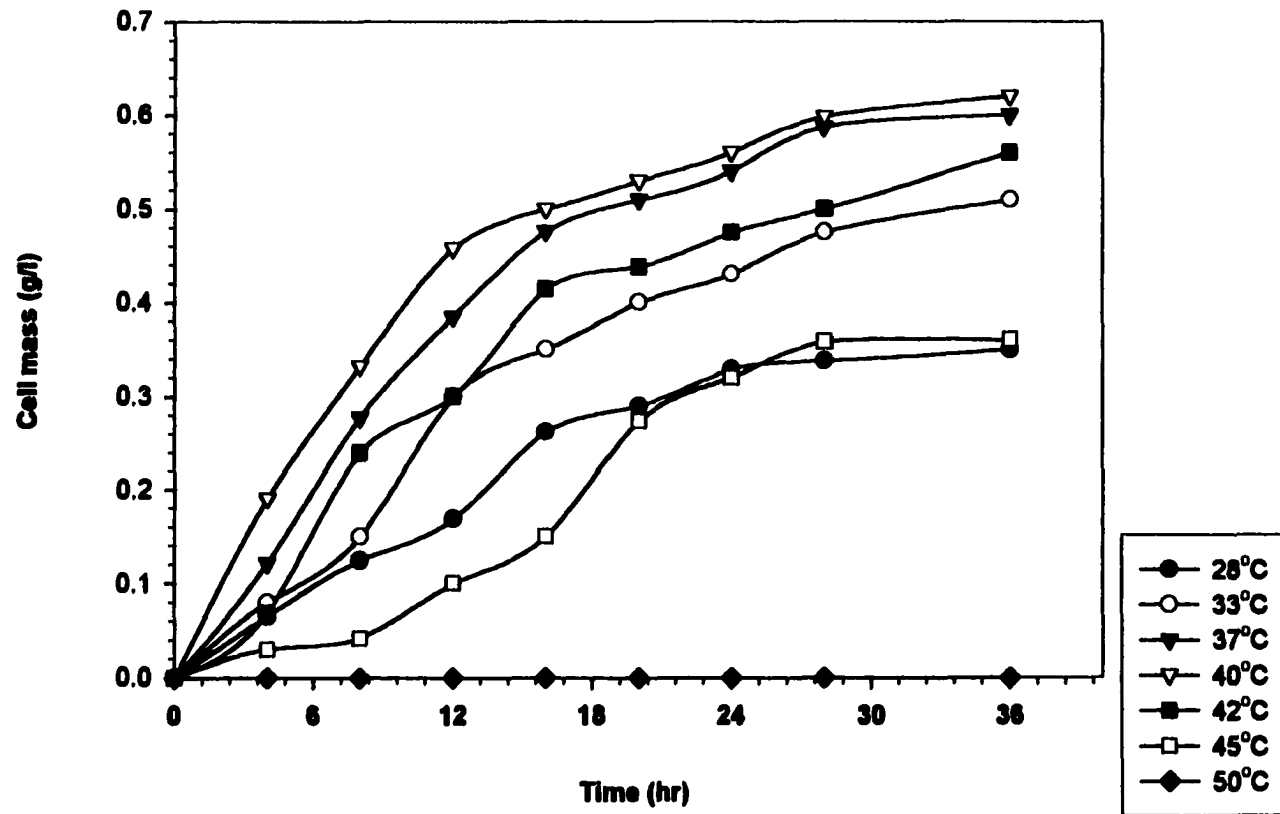


Figure 25. Growth in molasses of *L. casei* subsp. *rhamnosus* at various temperatures. Temperature tested were 28, 33, 37, 40, 42, 45 and 50°C. Molasses, 8% Brix, without nutrient supplementation was adjusted to pH 5.5 prior to use. These experiments were conducted as 100 ml cultures in 250 ml shake flasks at 200 rpm agitation rate. The shown values are the average values of duplicated experiments. The range values are in between 0 and 0.1.

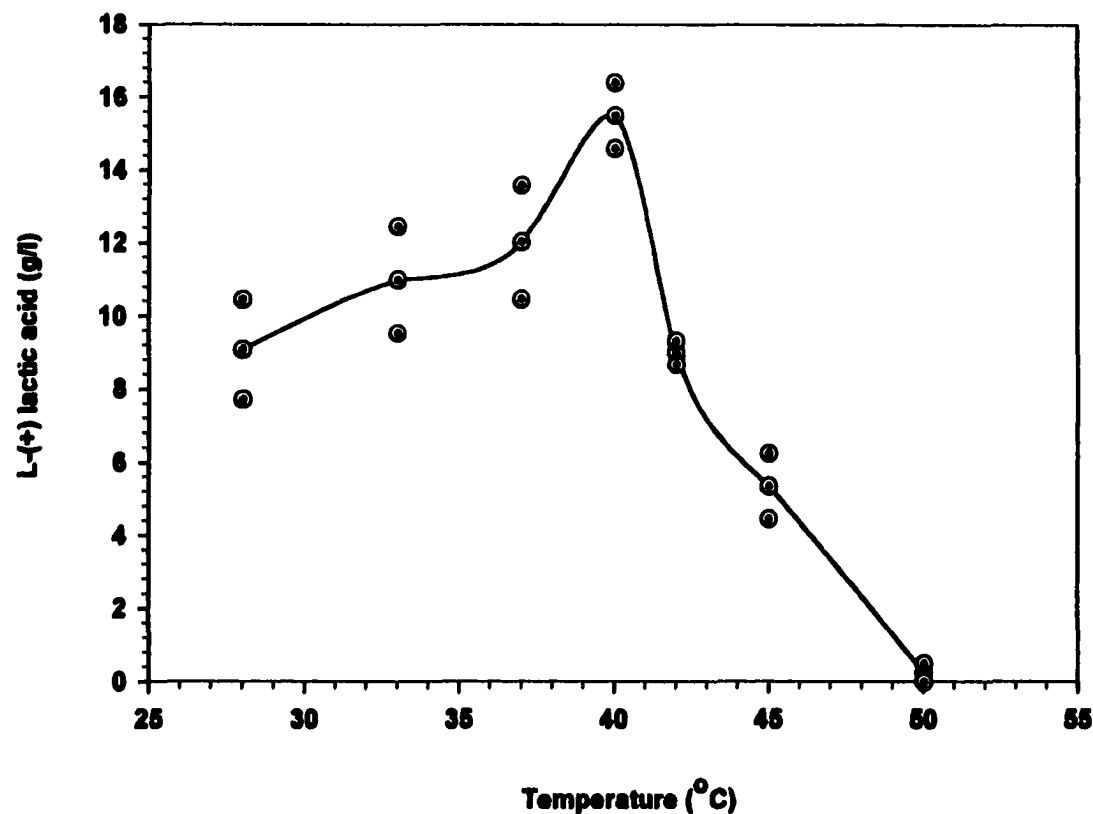


Figure 26. Effect of temperature on L-(+) lactic acid production by *L. casei* subsp. *rhamnosus* in molasses. Molasses, 8% Brix, without nutrient supplementation were adjusted to pH 5.5 prior to use. These experiments were conducted as 100 ml batch cultures in 250 ml Erlenmeyer flasks, without pH control. The agitation rate was 200 rpm. The range bars show the lowest and highest values of duplicated experiments.

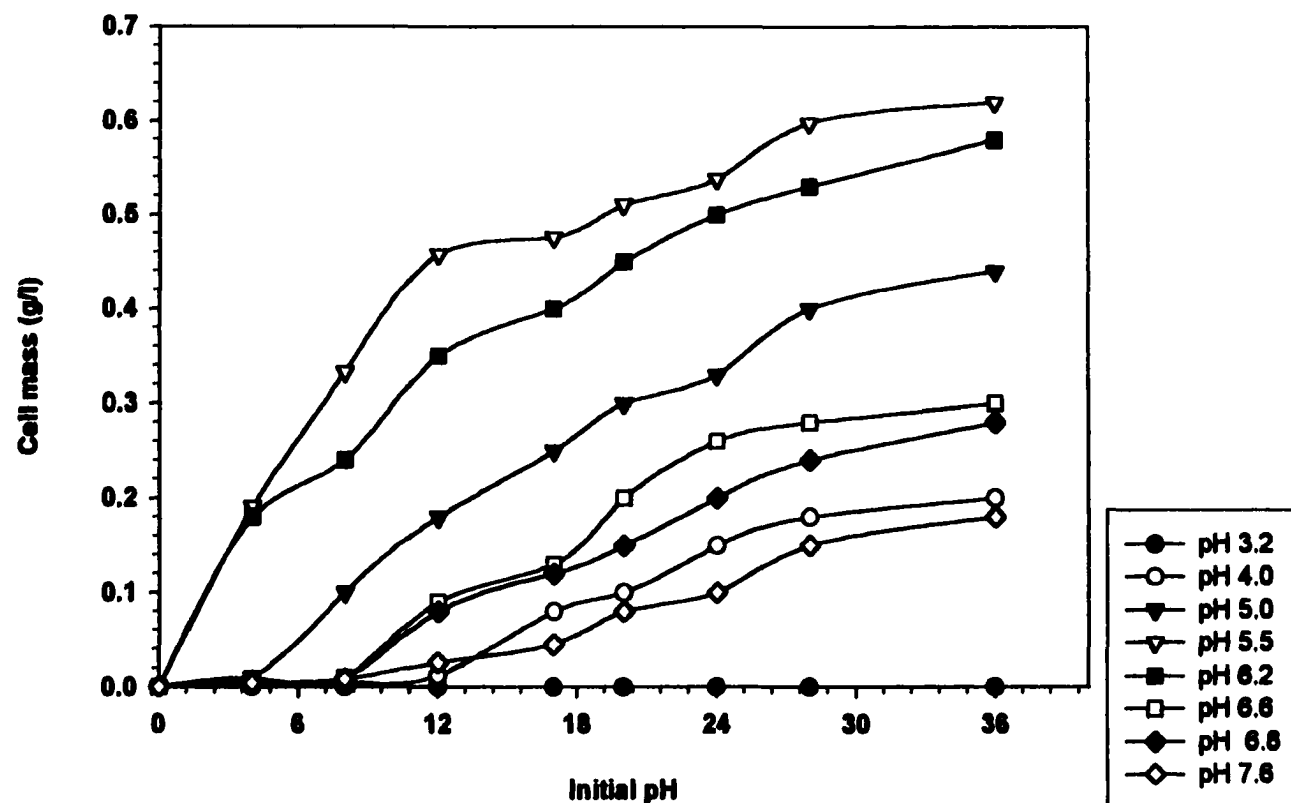


Figure 27. Growth of *L. casei* subsp. *rhamnosus* in molasses, 8% Brix, with time at different initial media pH's. These experiments were conducted in shake flasks, 100 ml working volume. Molasses media were not supplemented. Temperature was 40°C. The agitation rate was 200 rpm. The shown values are average values of duplicated experiments. The range values are between 0 and 0.1.

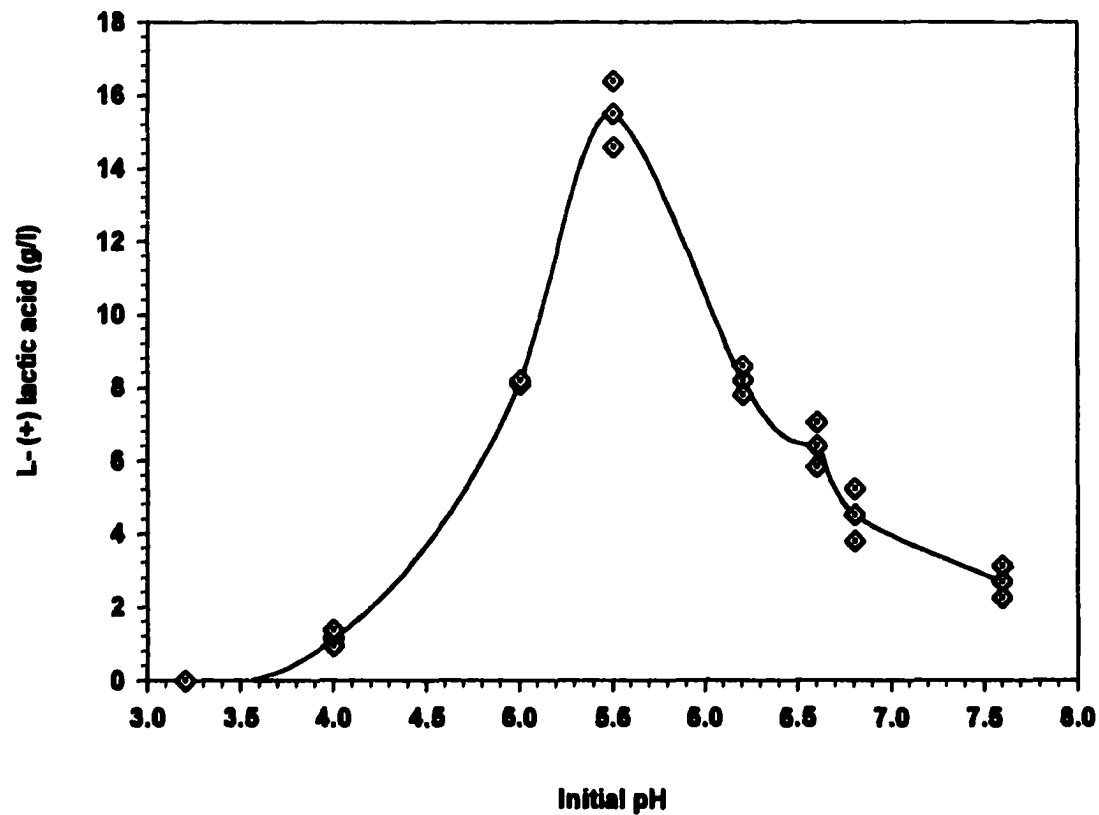


Figure 28. Effect of initial pH on lactic acid production of *L. casei* subsp. *rhamnosus*. These experiments were conducted in shake flasks, 100 ml working volume. Temperature was 40°C with 200 rpm agitation rate. Molasses media, without nutrient supplementation, were prepared to 8% Brix prior to use. There was no nutrient supplementation. Values were measured after 36 hours of growth. The range bars show the lowest and highest values of duplicated experiments.

high sugar content inhibited growth. In 1.8% Brix molasses (13 g/l total fermentable sugar content), 2.2 g/l of L- (+) lactic acid was produced with a molar conversion of 1.07 moles L- (+) lactic acid/mole hexose utilized. When molasses concentration was increased to 4, 7.3, 10.6 or 13.7% Brix, the conversion rates were 1.0, 0.69, 0.59 and 0.56 moles L-(+) lactic acid/mole hexose, with the lactate concentrations of 3.0, 3.4, 5.6 and 6.4 g/l, respectively. Residual sugars were observed. The higher the molasses concentration, the greater the concentration of residual sugars (Fig. 29). The ratio's of sucrose: glucose: fructose in the media were 0.75: 0.1: 0.15. After fermentation, the ratio of sucrose: glucose: fructose changed, all glucose was consumed but there were some residual fructose and sucrose. The amounts of residual sucrose and fructose were dependent on the starting molasses concentrations.

When L-(+) lactic acid (0, 0.04, 0.01, 0.24, 0.36, 0.44 and 0.61 moles/l) was added to 8 % Brix molasses, pH 5.5, growth of *L. casei* subsp. *rhamnosus* was inhibited. The growth inhibitory constant was 0.36 moles/l (32.4 g/l) (Fig. 30).

When sodium azide, 0, 7.69×10^{-4} , 1.54×10^{-3} , 7.69×10^{-3} , 0.015, 0.03 and 0.06 moles/l, was added to 8% Brix molasses media, growth of *L. casei* subsp. *rhamnosus* was inhibited. The inhibitory constant (K_i) was not determined as the growth inhibition was not directly proportional to concentration of the inhibitor (Fig. 31).

Molasses, 8% Brix, was supplemented with yeast extract (0.2, 0.4 and 0.8% w/v) and adjusted to pH 5.5 prior to inoculation. After 24 hours, L-(+) lactic acid concentrations were determined. Yeast extract enhanced bacterial growth by shortening growth lag phase. Molasses supplemented with 0.8% yeast extract produced higher cell mass than with 0.2 or 0.4% yeast extract. Cell mass from 0, 0.2, 0.4 and 0.8% supplements were 0.60, 0.69, 0.83 and 0.92 g/l, respectively (Fig. 32). However, the bacterial growth ceased when the pH dropped to where they could not grow any further (pH below 3.8). L-(+) lactic acid production was 7.5, 8.5, 8.9 and 9.9 for 0, 0.2, 0.4 and 0.8% yeast supplementation (Fig. 33). The ratio's of lactic acid produced to cell mass were 12.5, 12.3, 10.7 and 10.7 g/g, respectively.

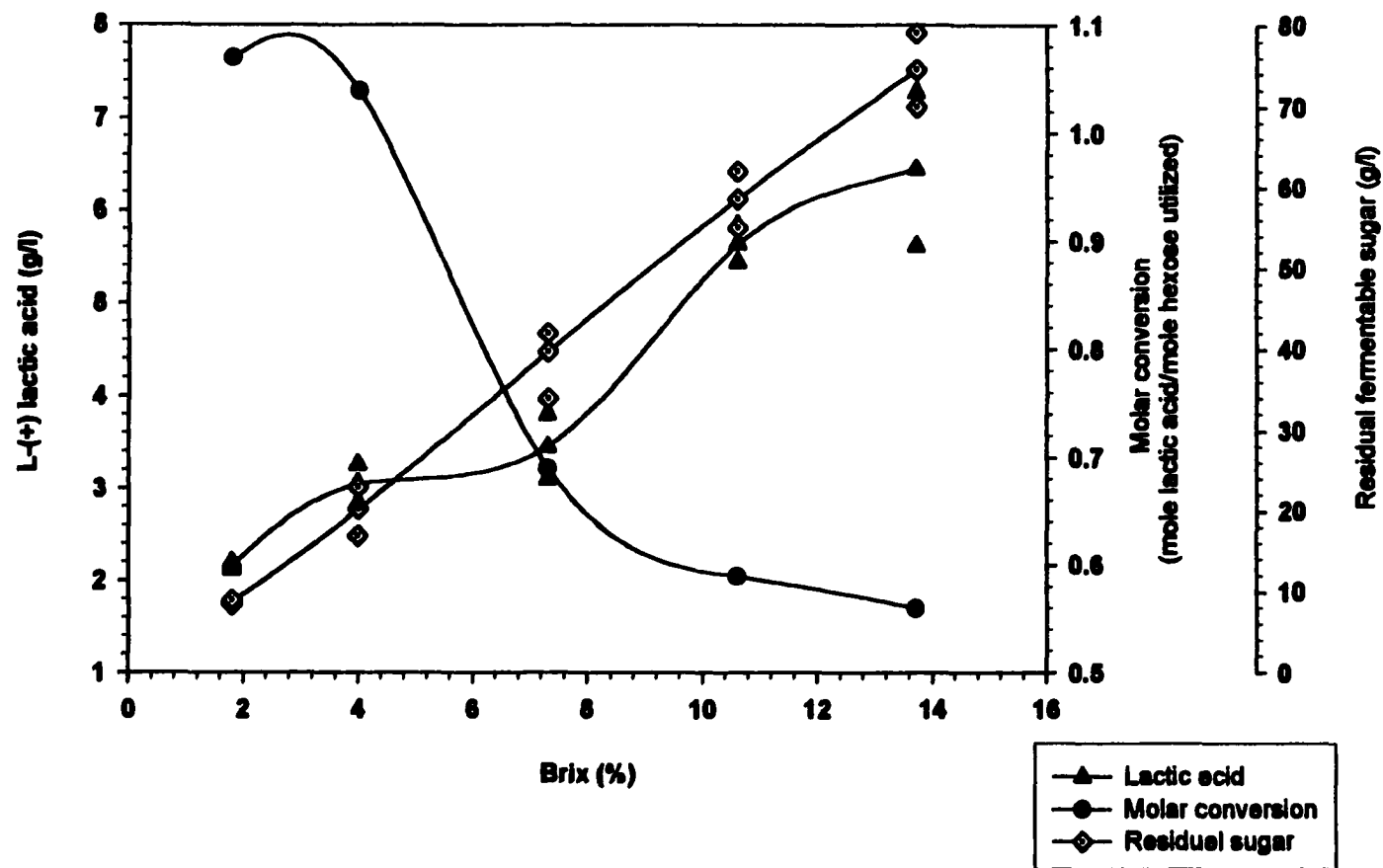


Figure 29. Effect of molasses concentration on L-(+) lactic acid production by *L. casei* subsp. *rharnosus*. These experiments were conducted in Erlenmeyer flasks without pH control, 40°C and 200 rpm agitation rate. The initial media pH was 5.5. Molasses media were not nutrient supplemented. Sugar refers to the sum of the sucrose, glucose and fructose present. The range bars show the lowest and highest points of duplicated experiments.

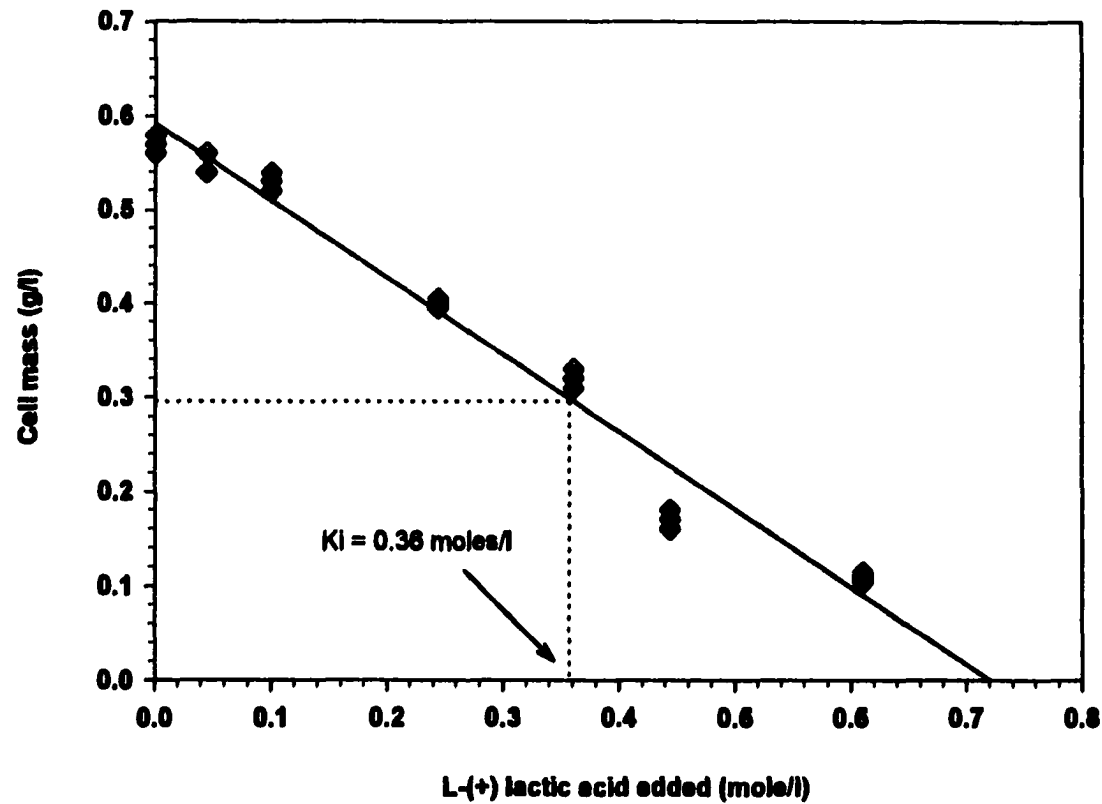


Figure 30. Inhibitory effect of L- (+) lactic acid on growth of *L. casei* subsp. *rhamnosus* in molasses, 8% Brix. These experiments were conducted in shake flasks with a working volume of 100 ml at 40°C, 200 rpm agitation rate. The starting pH was 5.5. Molasses was supplemented with 0, 0.04, 0.1, 0.24, 0.36, 0.44 or 0.61 M. The range bars show the lowest and highest values of duplicated experiments.

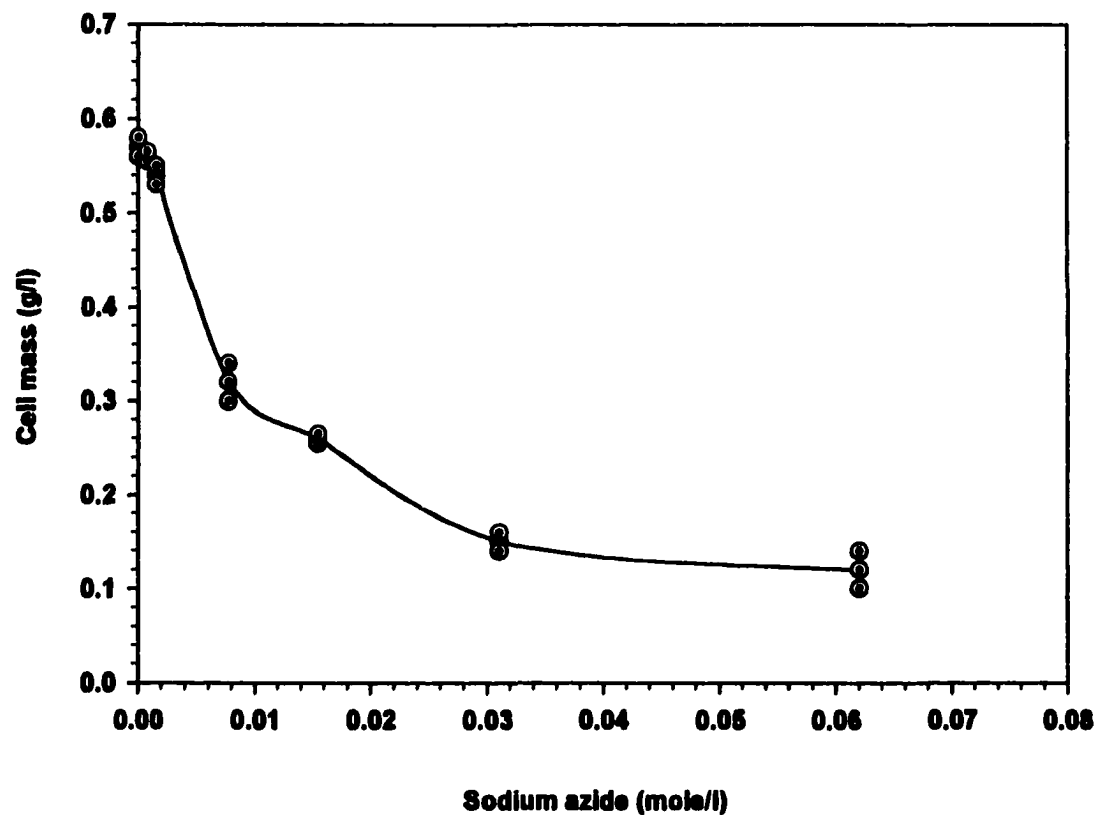


Figure 31. The growth inhibition of *L. casei* subsp. *rhamnosus* by sodium azide. These experiments were conducted in shake flasks at 40°C with 200rpm agitation rate. Molasses, 8% Brix, was supplemented with 0, 7.69×10^{-4} , 1.54×10^{-3} , 7.69×10^{-3} , 0.015, 0.031 or 0.06 M of sodium azide prior to use. The media were adjusted to pH 5.5. The range bars show the lowest and highest values of duplicated experiments.

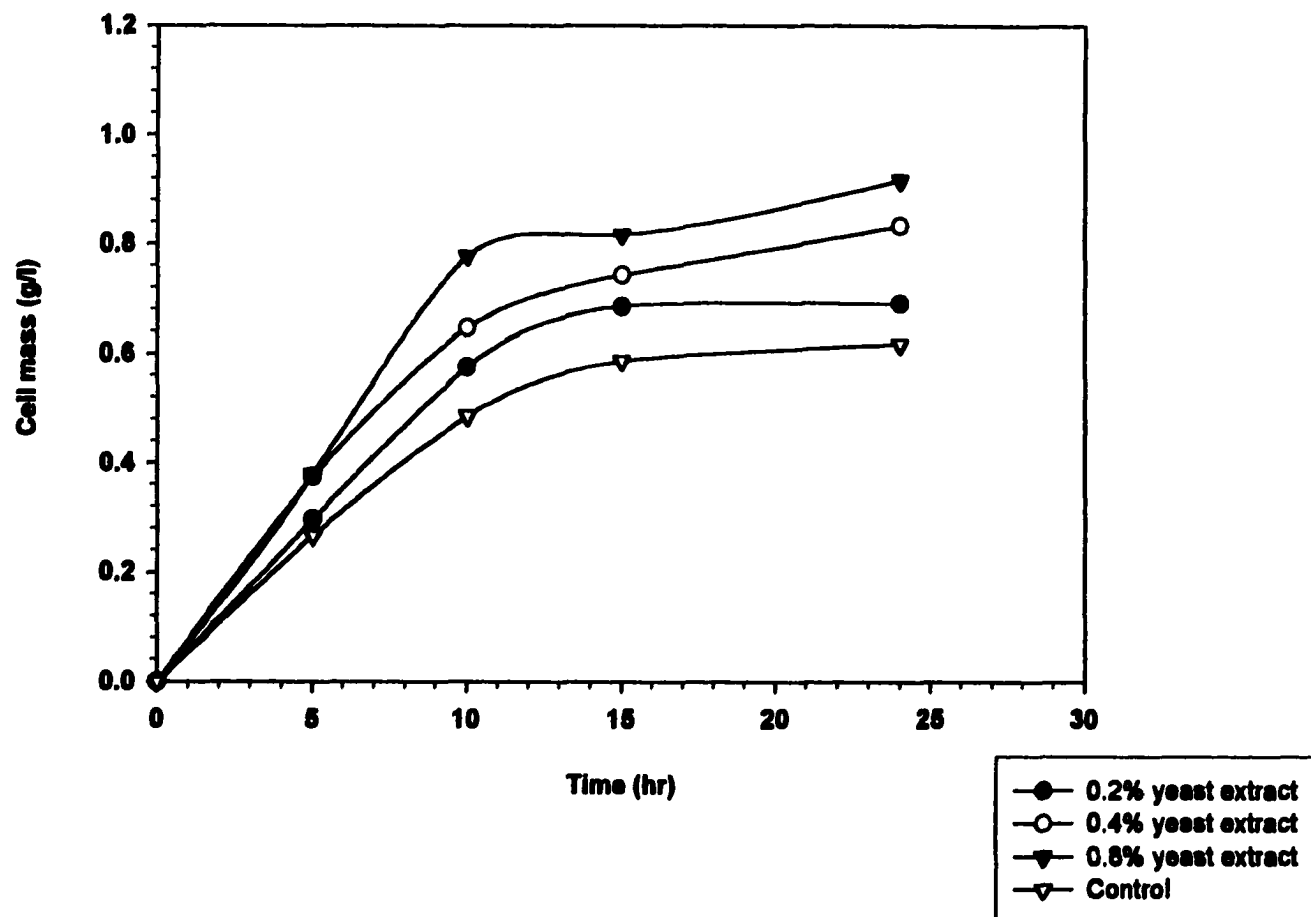


Figure 32. Effect of nutrient supplementation of molasses on growth of *L. casei* subsp. *rhamnosus*. Molasses, 8% Brix, was supplemented with 0, 0.2, 0.4 or 0.8% yeast extract. The media was adjusted to pH 5.5 prior to use. These experiments were conducted at 40°C, 200 rpm agitation rate, as 100 ml cultures in 250 ml shake flasks. The shown values are the average values of duplicated experiments. The range values are in between 0.05 and 0.2.

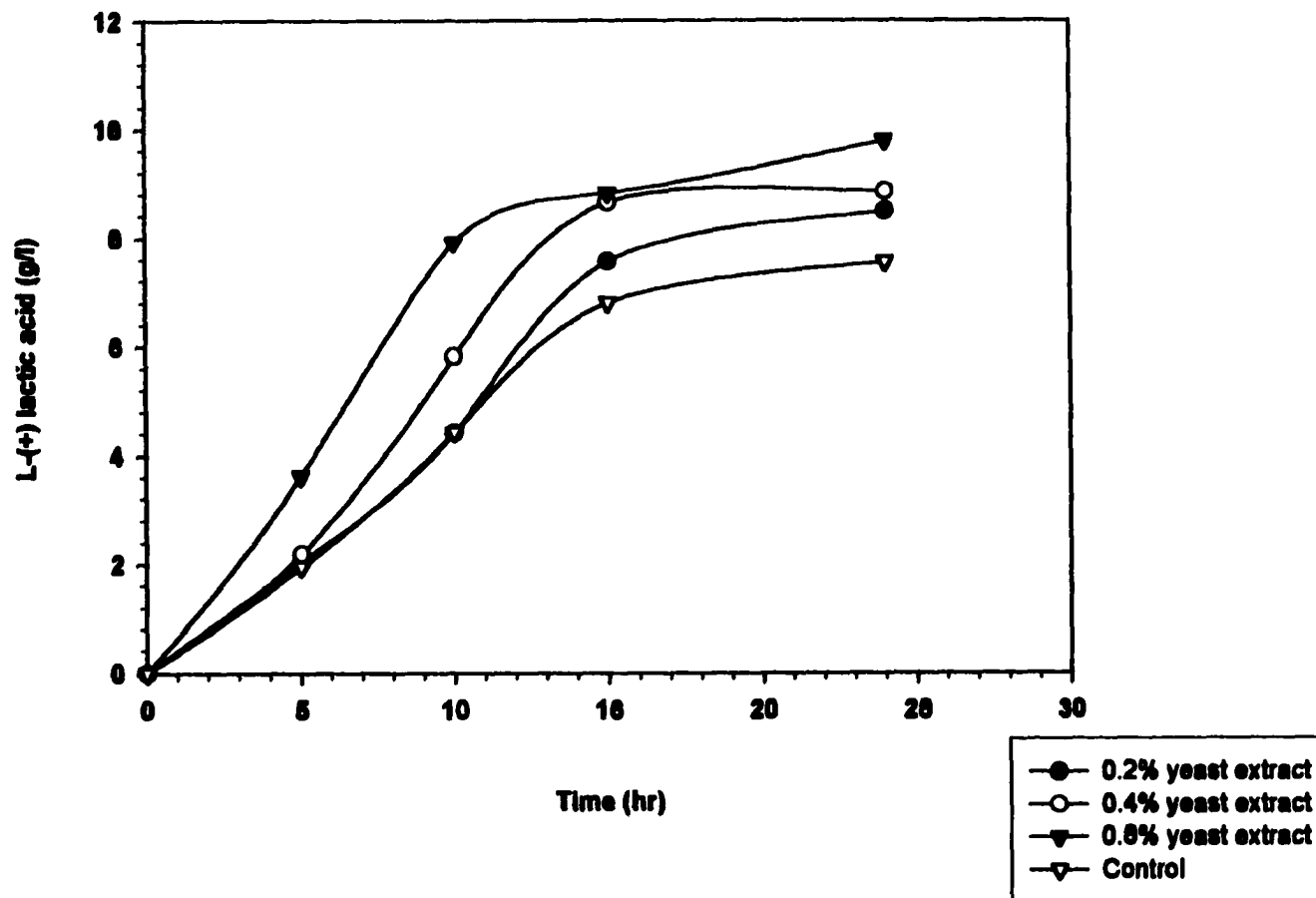


Figure 33. Effect of yeast extract concentrations on L-(+) lactic acid production by *L. casei* subsp. *rhamnosus* grown on molasses, 6% Brix. These experiments were conducted as 100 ml cultures in shake flasks without pH control at 40°C with 200 rpm agitation rate. The starting pH was 5.5. The shown values are average values of duplicated experiments. The range values are in between 0.5 and 1.0.

Lactobacillus casei subspecies *ramnosus* grew on blackstrap molasses with the slower rate when compared to MRS media. Table 5 summarizes the growth conditions of *L. casei* subsp. *ramnosus* on MRS and molasses media.

B. Fermentor studies (pH controlled)

B.1 Lactic acid production on *Lactobacillus* MRS media: In all fermentations using MRS media, all glucose in the fermentation broths were completely consumed within 14 to 72 hours depending on substrate concentrations. The initial glucose concentration of 60 g/l produced the highest productivity (3.8 g/l-hr). Increasing glucose concentrations to 100 and 120 g/l glucose reduced productivities to 2.9 and 2.1 g/l-hr, respectively (Fig. 34), but lactic acid concentrations were higher than in fermentations of 60 g/l glucose.

B.2 L-(+) lactic acid production on molasses

B.2.1 Effect of culture pH. Fermentations were conducted in molasses, 8% Brix, supplemented with 0.4% (w/v) yeast extract. The fermentations were controlled at a desired pH. The highest yield (87%) of L- (+) lactic acid (39 g/l) was produced at pH 5.5. The lowest concentration of L- (+) lactic acid was produced (3.5 g/l) with a substrate conversion of 6%, when the culture pH was 4. Fermentation at all pH values, except pH 5.5, did not go to completion (Fig. 35).

B.2.2 Effect of molasses concentration. Fermentations were conducted in the following molasses concentrations (4, 8, 14, 19, 23 and 30 % Brix) without additional nutrient supplementation. The most L-(+) lactic acid (28 ± 2 g/l) was produced when the total sugar concentration was 47 ± 3 g/l (8% Brix) with a yield of 1.2 moles lactic acid/moles hexose utilized (Fig. 36). Increasing sugar concentration beyond this point did not increase L- (+) lactic acid yield. Lowering molasses concentration to 4% Brix resulted in higher molar conversion (1.46 moles lactic acid/moles hexose), but lower lactic acid production (12 ± 1 g/l). Duration of fermentation was not affected by molasses concentration, with all fermentations finishing

Table 5. Summary of shake flask studies on lactic acid production from MRS media and blackstrap molasses by *L. casei* subsp. *rhamnosus*.

| Condition | MRS media | Molasses |
|---------------------------------------|-----------|----------------|
| Temperature (°C) | 40 | 40 |
| Media pH | 5.5 | 5.5 |
| Sugar substrate concentration (g/l) | 20-60 | 80 |
| Lactic acid inhibition constant (g/l) | 11 | 32 |
| Sodium azide inhibition constant (mM) | 2 | Not determined |

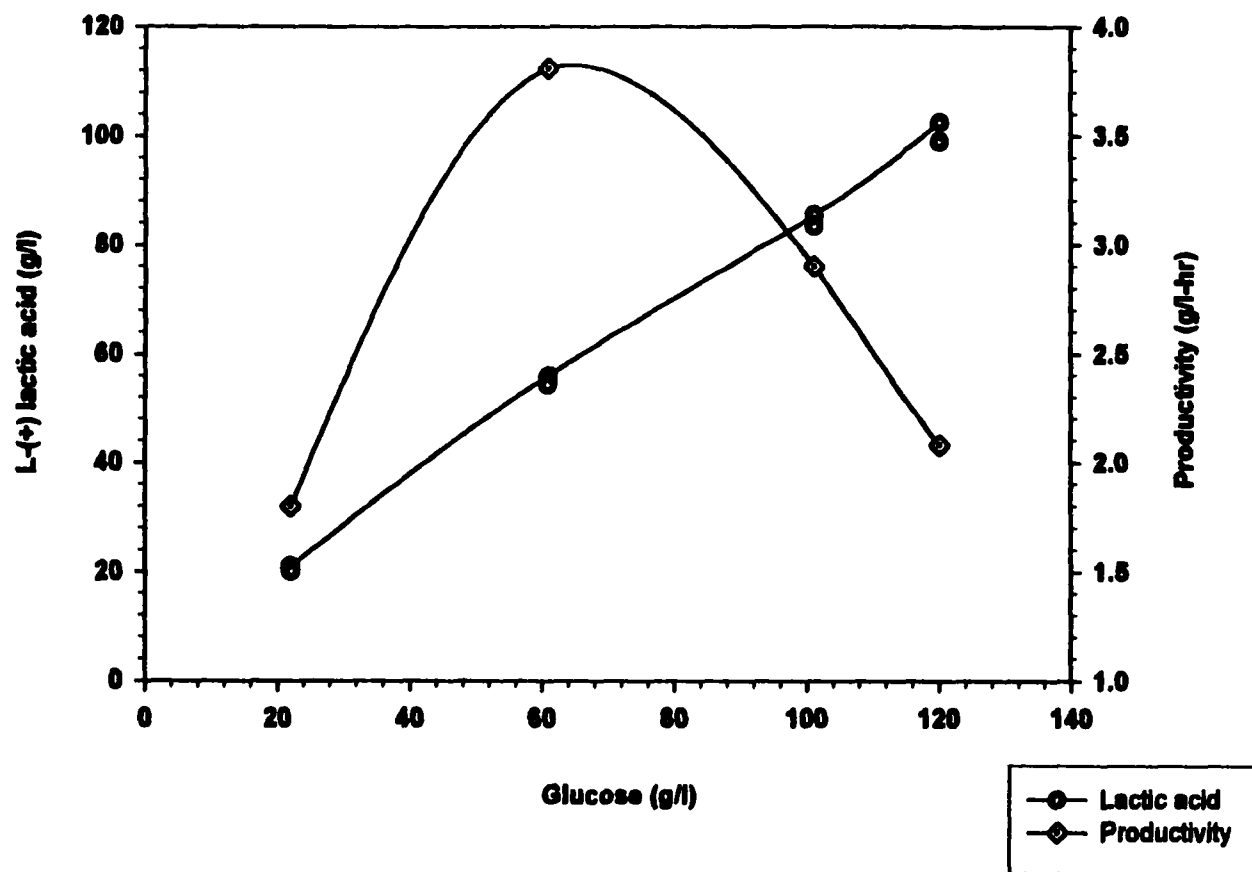


Figure 34. L-(+) lactic acid production on *Lactobacillus* MRS media by *L. casei* subsp. *rhamnosus*. These experiments were conducted in 1 liter fermenters at 40°C with 200 rpm agitation rate and were pH controlled at 5.5. The range bars show the lowest and highest values of duplicated experiments.

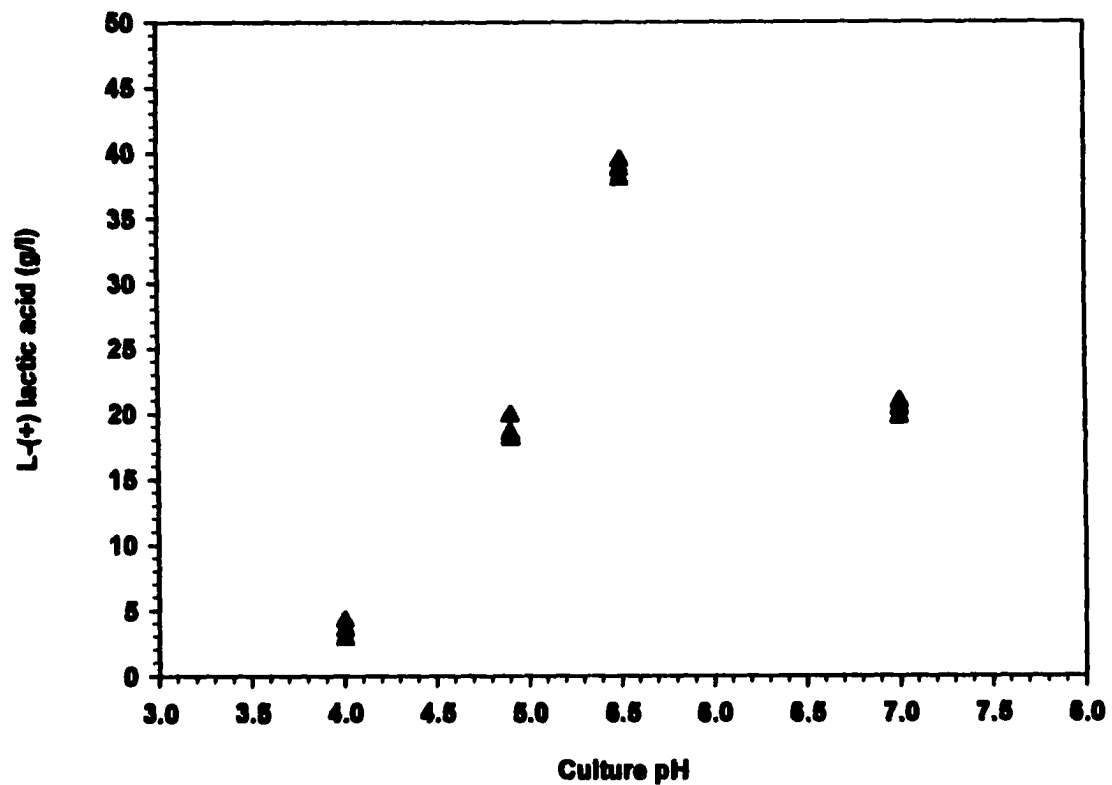


Figure 35. Effect of culture pH on L- (+) lactic acid production by *L. casei* subsp. *rhamnosus*. These experiments were done in fermenters (1 liter) with pH control. Molasses media, 5% Brix, were supplemented with 0.4% yeast extract. Temperature was 40°C and the agitation rate was 200 rpm. L-(+) lactic acid production was monitored until concentration increase stopped, normally about 72 hours. The range bars show the average, lowest and highest values of duplicated experiments.

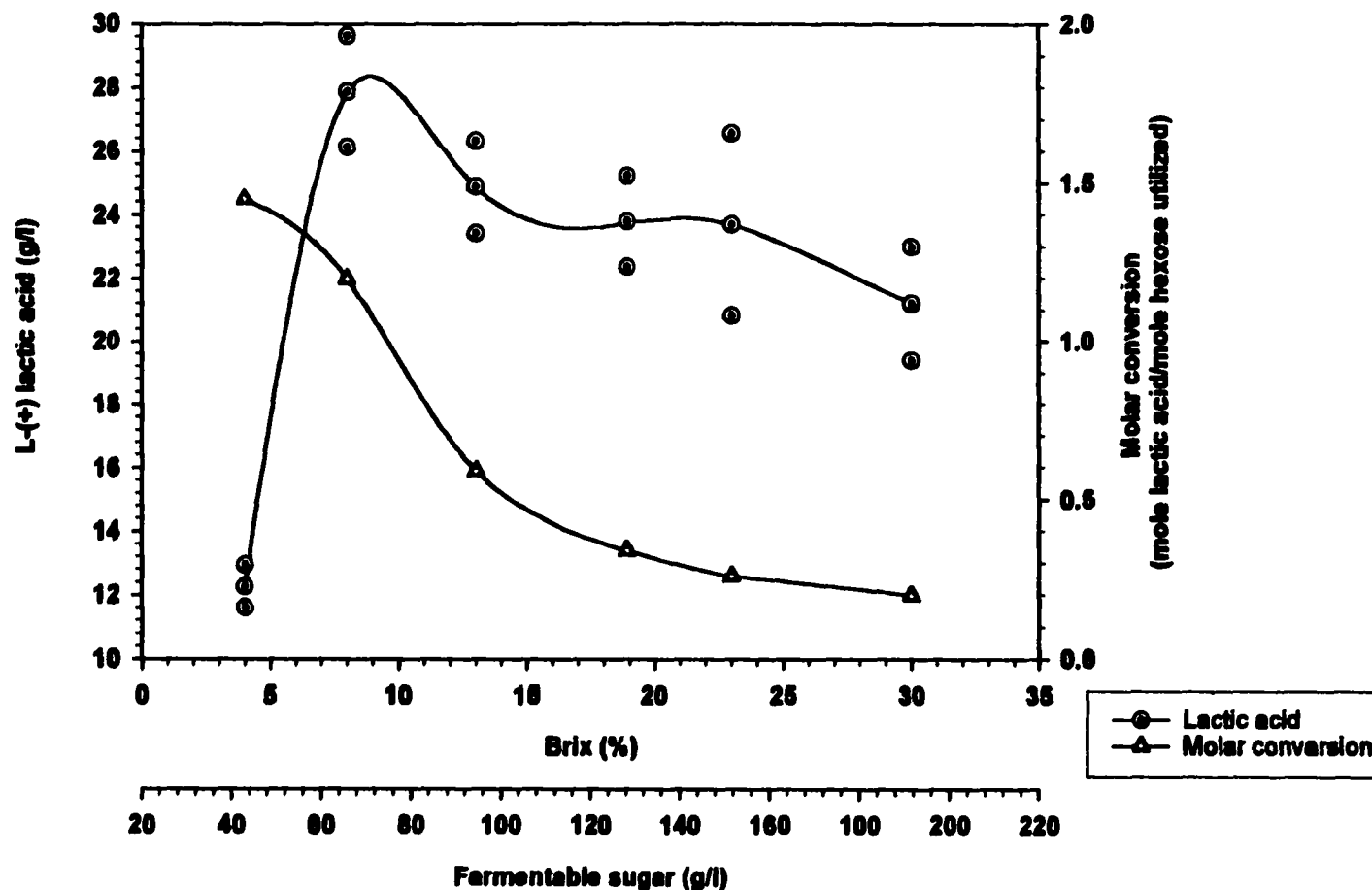


Figure 36. Effect of sugar concentration on batch fermentation of lactic acid production and molar conversion by *L. casei* subsp. *rhamnosus*. These experiments were conducted using various concentrations of molasses without nutrient supplementation in 1 liter fermenters. Fermentations were controlled at pH 5.5, 40°C and 200 rpm agitation rate. The range bars show the lowest and highest values of duplicated experiments. Molar conversions were calculated from the mean values for each condition.

around 72 hours post inoculation. When the fermentation was pH controlled, lactic acid was produced approximately 5 times higher than from no pH controlled fermentations.

B.2.3 Effect of molasses clarification on fermentations. Molasses were clarified as described in the Materials and Methods. When clarified molasses (8% Brix, pH 5.5) were used as media, L- (+) lactic acid production decreased 53% (13.5 ± 2 g/l) compared to the untreated molasses (28 ± 2 g/l). The molar conversion dropped to 0.47 moles lactic acid/moles hexose utilized (Table 6). The productivity of lactic acid production from clarified molasses was 0.2 g/l-hr. However, when heat inactivated invertase, 1% w/v, was added into 8% Brix clarified molasses, production increased to 27 ± 0.7 g/l lactic acid. The productivity was 0.38 g/l-hr. Residual sugars were observed in all cases, except for the control which had no supplementation or inversion. This suggests that the protein preparation provided some missing nutrients for *L. casei* subsp. *rhannosus* growth.

B.2.4 Effect of molasses inversion on fermentation

When the sucrose in molasses was inverted by reducing the pH with sulfuric acid and heating for 4 hours prior, the inversion was not complete unlike with invertase inversion (see below). L-(+) lactic acid production from acid inverted molasses was the same as was found for clarified molasses (13.5 ± 2 g/l) (Table 6).

Molasses where the sucrose had been inverted by invertase produced more L- (+) lactic acid. Invertase (1% w/v) completely inverted the sucrose in molasses to glucose and fructose at pH 4.5, 56°C after 20 minutes of incubation (Fig.37). L- (+) lactic acid production from inverted molasses, 8% Brix, was 41 ± 1.2 g/l, a 91 ± 3 % yield. The productivity of L- (+) lactic acid production from inverted molasses was 1.0 g/l-hr. The molar conversion was 1.8 ± 0.1 moles lactic acid/moles hexose utilized, which was significantly higher than the conversion from either non-supplemented, untreated molasses (1.2 moles/moles) or clarified molasses (0.47 moles/moles) (Fig.38). When invertase was decreased from 1 to 0.5 and 0.2% (w/v), the inversion of the sucrose in molasses was complete, but the treatment time had to be extended to 1 hour. L-(+) lactic acid production from 0.2 and 0.5% (w/v) invertase treated molasses were

Table 6. Effect of molasses treatment on L- (+) lactic acid production by *L. casei* subsp. *rharmnosus*.

| Batch | Initial sugar (g/l) | | | Residual sugar (g/l) | | | L-(+) lactic acid (g/l) |
|----------------------------|---------------------|---------|----------|----------------------|---------|----------|-------------------------|
| | Sucrose | Glucose | Fructose | Sucrose | Glucose | Fructose | |
| Control | 32±6 | 6.5±3. | 7.5±3 | 0 | 0 | 0 | 28±2 |
| Clarified | 41±6 | 6 | 8.4±0.2 | 27±6 | 0.3±0.4 | 0.15±0.2 | 13.5±2 |
| Protein added ^a | 31±4 | 4±0.5 | 6 | 5.0±0.2 | 0 | 0.2±0.1 | 27±0.7 |
| Acid inverted ^b | 15±2 | 13±0.5 | 15.0±0.5 | 13.0±0.4 | 6±0.5 | 10±0.5 | 14±1.2 |

Control was molasses with neither treatment nor nutrient supplementation. Clarified molasses, 8% Brix, were not supplemented. ^a, molasses was supplemented with a heat inactivated invertase, 1%w/v. ^b, molasses, 8% Brix, was reduced pH to pH 3 and was heated for 4 hours. These experiments were conducted in fermenters. The molasses, with no nutrient supplementation, was adjusted to pH 5.5 prior to use. The fermentations were controlled at pH 5.5, 40°C and 200 rpm agitation rate. The fermentations were allowed to run until there was no further increase in lactic acid concentration, normally about 72 hours. Values are average of two repeated experiments.

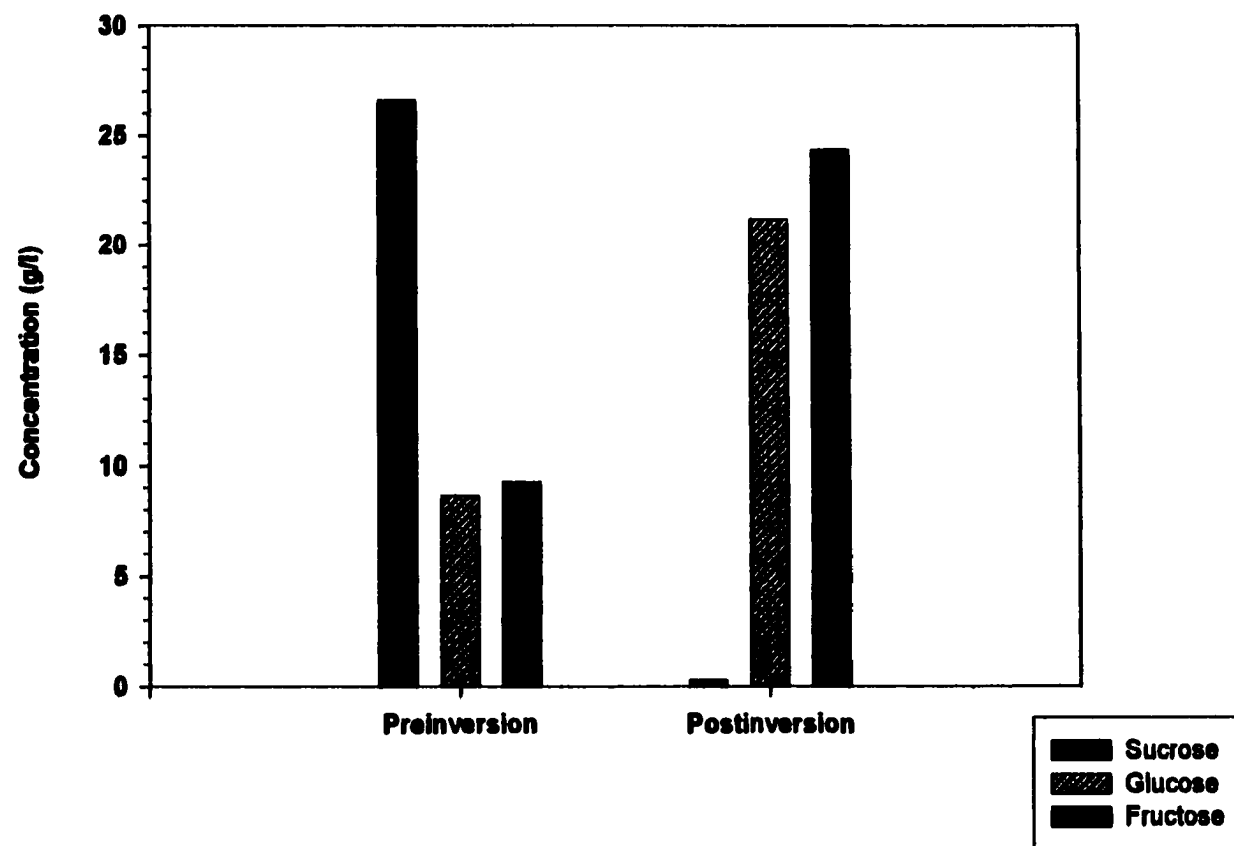


Figure 37. Molasses inversion by invertase. Invertase (1% w/v) was added into molasses, 8% Brix, pH 4.5, and was incubated at 56°C for 20 minutes. The shown values are the average values from duplicated experiments. The range values for sucrose, glucose and fructose are 0.5, 0.9 and 0.7 for the preinversion, and 0.4, 1.9 and 2.6 for postinversion, respectively.

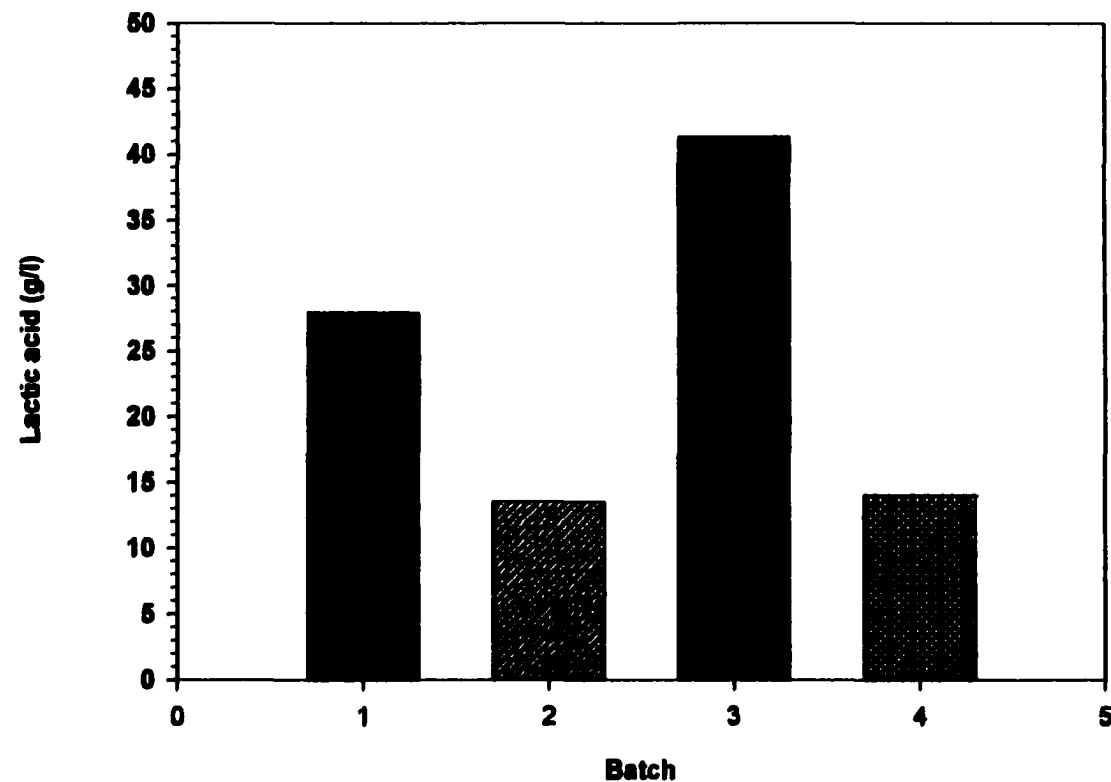


Figure 38. Batch fermentations by *L. casei* subsp. *ramnosus* on treated and untreated molasses, 8% Brix, at pH 5.5, 40°C and 200 rpm agitation rate. Batch 1, molasses were not treated. Batch 2, molasses were clarified. Batch 3, molasses were inverted with 1% invertase. Batch 4, molasses was acid inverted. No additional nutrients were added. The fermentations were allowed to go to completion, usually about 72 hours. The shown values are the average values of duplicated experiments. The range values are 1.8, 2, 1.2 and 1.2 for batch 1,2,3 and 4, respectively.

50 and 48 g/l, respectively (Fig. 39). The productivities were 1.2 and 1.0 g/l-hr for 0.2 and 0.5% invertase treated molasses, respectively.

B.2.5 Molasses supplementation. Yeast extract was the chosen nutrient supplement. With no supplementation, 28 ± 2 g/l L- (+) lactic acid was produced, with a molar conversion of 1.2 moles lactic acid/moles hexose. When 0.2% (w/v) yeast extract was added to molasses, 8% Brix, 40 g/l L- (+) lactic acid was produced with a 1.9 molar conversion. Increasing the yeast extract to 0.4% (w/v) resulted in the same amount of lactic acid produced (38 g/l) with the molar conversion of 1.6. At 0.8% yeast extract supplementation, 40 g/l L- (+) lactic acid was produced with 1.6 molar conversion. The fermentation times from these batches were different. At 0.8% yeast extract, the fermentation was complete after 42 hours while the fermentation time for 0.2 and 0.4% yeast extract supplemented molasses were 110 and 96 hours, respectively. Non-supplemented molasses was fermented completely after 120 hours. Fermentable sugars in both non-supplemented and supplemented molasses were completely utilized. The productivities were 0.2, 0.4, 0.4 and 0.9 g/l-hr for 0, 0.2, 0.4 and 0.8% yeast extract supplementation, respectively. Yeast extract improved L- (+) lactic acid productivity by shortening the lag phase of growth. Cell mass from 0.8% yeast extract supplemented molasses fermentations were also higher than from those with 0, 0.2 and 0.4% yeast extract. The ratio's of lactic acid produced to cell mass decreased when the concentrations of yeast extract increased; 81, 79, 51 and 31 g/g for 0, 0.2, 0.4 and 0.8% yeast extract supplementation, respectively (Table 7).

When the concentration of molasses supplemented with 0.4% yeast extract was 4% Brix, the molar conversion was 1.8 with L- (+) lactic acid concentration of 28 ± 2 g/l. At 8% Brix, 38 ± 1 g/l of L- (+) lactic acid was produced with a 1.6 molar conversion. Fermentations of 4 and 8% Brix molasses went to completion. When the molasses concentration was 13% Brix, 59 g/l of L- (+) lactic acid was produced with a molar conversion of 1.9 moles lactic acid/moles hexose. The highest L- (+) lactic acid concentration, 70 g/l, was observed from 18 % Brix molasses with a molar conversion of 1.6. Residual sugar was detected in all fermentations

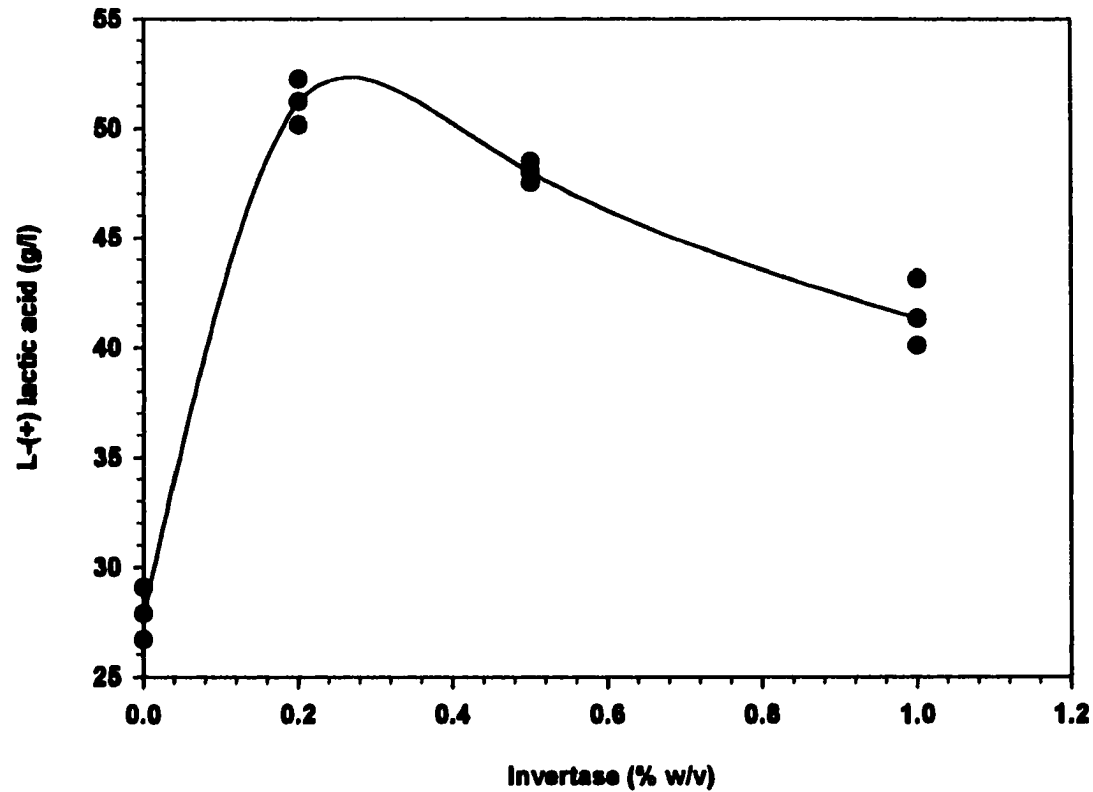


Figure 39. Effect of invertase concentrations on L-(+) lactic acid production by *L. casei* subsp. *rhamnosus*. These experiments were conducted in 1 liter fermenters at 40°C, 200 rpm agitation rate and pH 5.5. Molasses (8% Brix, pH 4.5) were added 0, 0.2, 0.5 and 1% (w/v) invertase, and incubated at 56°C for up to 1 hour based on invertase concentrations. The range bars show the lowest and highest values of duplicated experiments.

Table 7. Effect of yeast extract on lactic acid production by *Lactobacillus casei* subspecies *rhamnosus*.

| Yeast extract (%) | Initial sugar (g/l) | L-(+) lactic acid (g/l) | Molar conversion (moles lactic acid/moles hexose) | Productivity (g/l-hr) | Cell mass (g/l) | Lactic acid/cell mass (g/g) |
|----------------------|------------------------|----------------------------|--|--------------------------|--------------------|--------------------------------|
| 0 | 46±3 | 28±2 | 1.2 | 0.2 | 0.4 | 81 |
| 0.2 | 43 | 39.7 | 1.9 | 0.4 | 0.5 | 79 |
| 0.4 | 47±1 | 39±1 | 1.6 | 0.4 | 0.8 | 51 |
| 0.8 | 47±1 | 37±3 | 1.6 | 0.9 | 1.3 | 31 |

The experiments were conducted in 1 liter fermenters with pH control at pH 5.5, 40°C and 200 rpm agitation rate. Molasses, 8% Brix, were not treated with heat or an enzyme. Values are average numbers of two repeated experiments.

where the molasses concentration was above 8% Brix (Table 8). Batch fermentation of lactic acid production was sum up in Table 9.

C. Immobilized cell fermentation

C.1 Solid supports for cell immobilization: electron microscopy:

Alginate beads, polystyrene, polyurethane, perlite and activated carbon were tested as possible supports for cells. *L. casei* subsp. *rharmnosus* successfully immobilized in calcium alginate beads. However, the alginate beads dissolved after few days of operation at 40°C.

When activated carbon was used as a solid support, 5.6 ± 0.1 g/l lactic acid was produced from a media containing 3% sucrose supplemented with yeast extract and tryptone (0.5% w/v each). For perlite packed reactor, to 5.0 ± 0.3 g/l lactic acid was produced on the same media, but with a longer lag phase than that of activated carbon packed column. Only 3.3 ± 0.1 and 3.6 g/l lactic acid were produced from polystyrene and polyurethane foam packed bioreactors, respectively (Fig. 40). Activated carbon showed higher numbers of immobilized cells (200×10^3 CFU's/mm²) compared to perlite (6×10^3 CFU's/mm²), polyurethane foam (0.6×10^3 CFU's/mm²) and polystyrene foam (20×10^3 CFU's/mm²) as revealed by scanning electron microscopy (Fig. 41a, b, c and d) after 3 weeks of operation in an immobilized column.

C.2 Adsorption of lactic acid by activated carbon

Adsorption of L- (+) lactic acid was tested in order to determine whether there was an adsorption of lactic acid by granular activated carbon. Per gram of granular activated carbon, 8 ± 2 , 8 ± 2 and 8 ± 1 mg L- (+) lactic acid were absorbed at solution pH values of 4, 5 and 6, respectively (Fig. 42).

C.3 Immobilized cell bioreactors (10 ml):

In order to optimize the growth temperature of *L. casei* subsp. *rharmnosus* immobilized onto activated carbon, seven temperatures were tested; 38, 40, 42, 44, 46, 48 and 50°C. At 40°C, the immobilized culture produced the most L- (+) lactic acid, 3.2 g/l from 42 g/l sugar in molasses, at a dilution rate of 1.1/hr (Fig. 43). Decreasing the temperature to 38°C, less lactic

Table 8. Batch fermentations, by *L. casei* subsp. *rhannosus*, at various concentrations of molasses

| Brix (%) | 4 | 8 | 13 | 18 |
|--|-------------|---------------|---------------|--------------|
| Starting substrate concentrations | | | | |
| • Sucrose | 24±1 | 39±0.5 | 58±1.8 | 94±2 |
| • Glucose | 2±1 | 3±0.4 | 4±1 | 7±0.8 |
| • Fructose | 3±1 | 5±1 | 7±3 | 8±3 |
| L- (+) lactic acid (g/l) | 28±1 | 39±0.7 | 57±1 | 71±1 |
| Molar conversion | 2 | 1.6 | 1.9 | 1.6 |
| Yield (%): Total sugars | 92 | 78 | 78 | 61 |
| Duration (hr) | 85 | 96 | 174 | 115 |
| Productivity (g/l-hr) | 0.3 | 0.4 | 0.34 | 0.5 |
| Residual sugars (g/l) | | | | |
| • Sucrose | 0 | 0 | 11 | 26 |
| • Glucose | 0 | 0 | 0 | 0.2 |
| • Fructose | 0 | 0 | 0.2 | 0.4 |

Media were supplemented with 0.4% yeast extract. The fermentations were conducted at 40°C at 200 rpm agitation rate and pH 5.5. Sugar and lactic acid concentrations are shown as average values of two repeated experiments. Molar conversion refers to moles of lactic acid produced/moles of hexose utilized.

Table 9. Summary of lactic acid production from blackstrap molasses by *L. casei* subsp. *rhamnosus* by batch fermentations.

| Condition | Result |
|--|--|
| Temperature (°C) | 40 |
| Culture pH | 5.5 |
| Molasses concentration (% Brix) | 8 |
| Nutrient supplementation ^a | 0.2% invertase |
| Lactic acid concentration (g/l) ^b | 50 |
| Residual fermentable sugar (g/l) | 0 |
| Clarified molasses | 53% decrease in lactic acid production |
| Acid inverted molasses | 53% decrease in lactic acid production |

^a, the most efficient condition for batch fermentations from this study. ^b, lactic acid production from 8% Brix molasses supplemented with invertase, 0.2% (w/v).

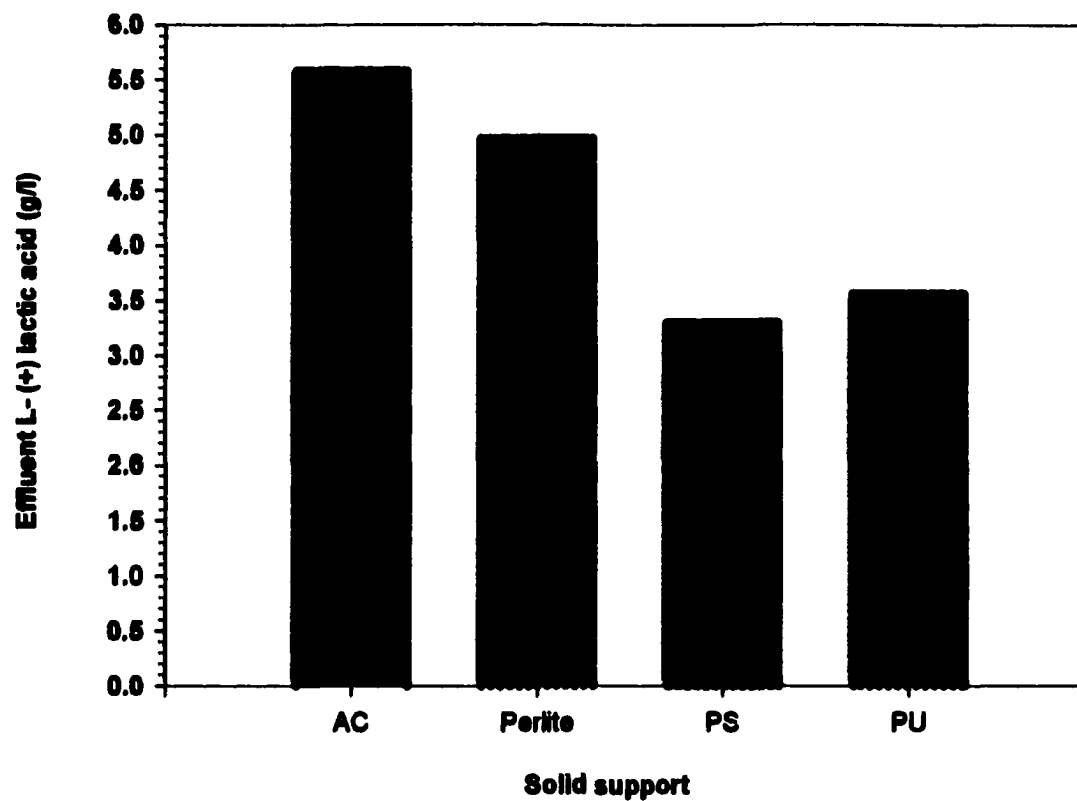
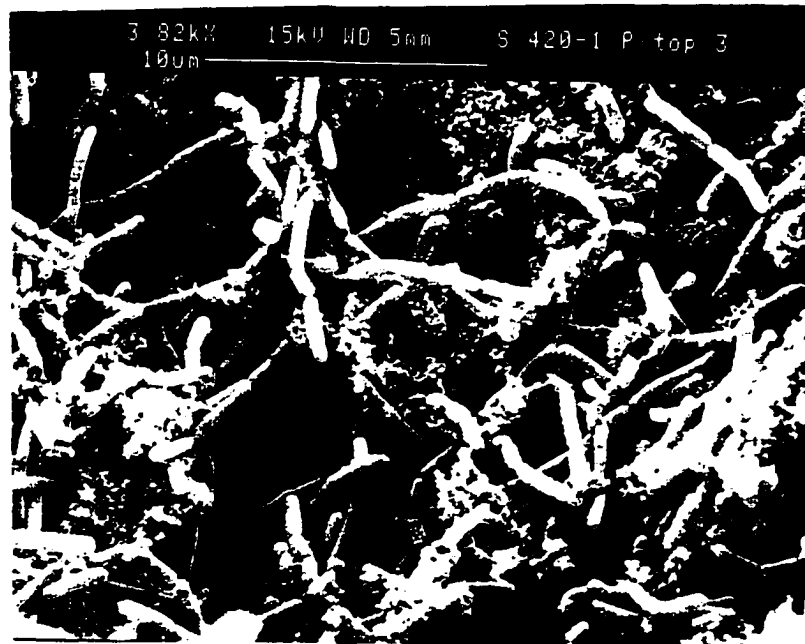


Figure 40. L- (+) lactic acid yield as a function of support using immobilized *L. casei* subsp. *rhannosus*. These experiments were conducted at 40°C. Media, pH 5.5, containing 3% sucrose, yeast extract and tryptone (1% each) were fed into columns at a flow rate of 6 ml/hr (0.6/hr dilution rate). AC, activated carbon; PS, polystyrene foam; PU, polyurethane foam. The columns were allowed to stabilize for 4 days prior to measurement. The shown values are the average values of duplicated experiments. The range values of activated carbon, perlite, polystyrene and polyurethane are 0.1, 0.3, 0.1 and 0.03, respectively.

(a)



(b)

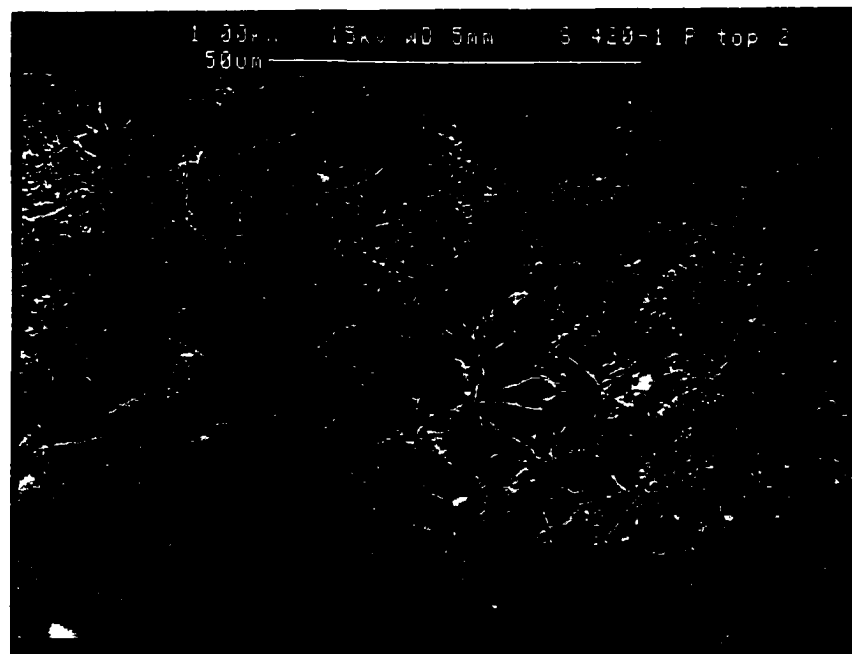
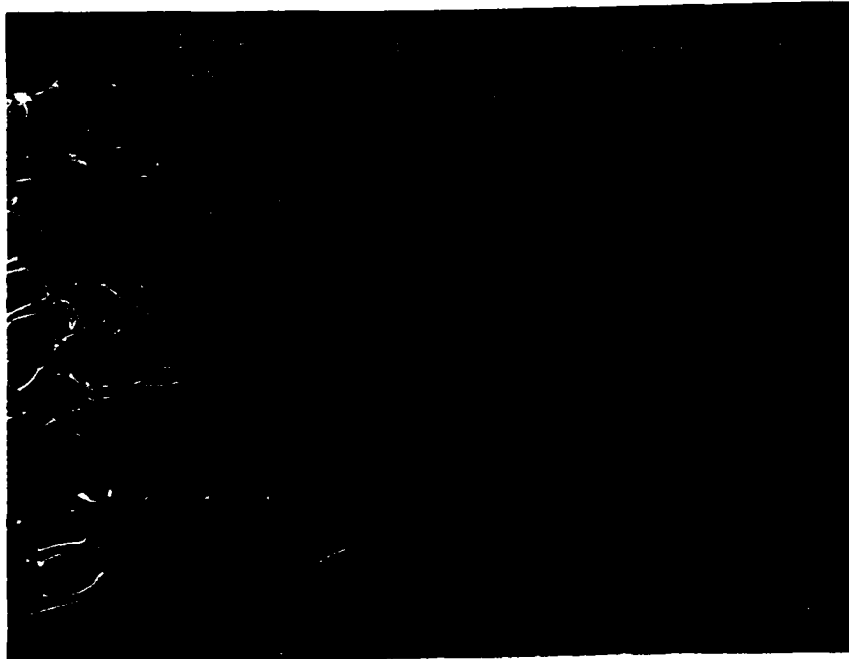


Figure 41a. SEM photograph of *Lactobacillus casei* subspecies *rhamnosus* immobilized onto granular activated carbon. (a) The bar represents 10 μm at a 3820x magnification. (b) The bar represents 50 μm at a 1000x magnification. The cultures were three weeks old.

(a)



(b)

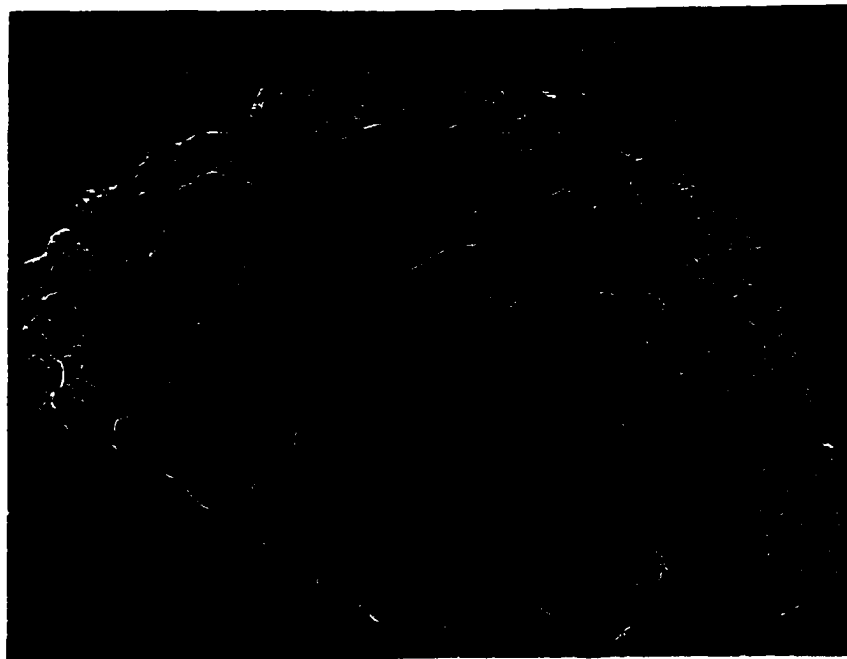
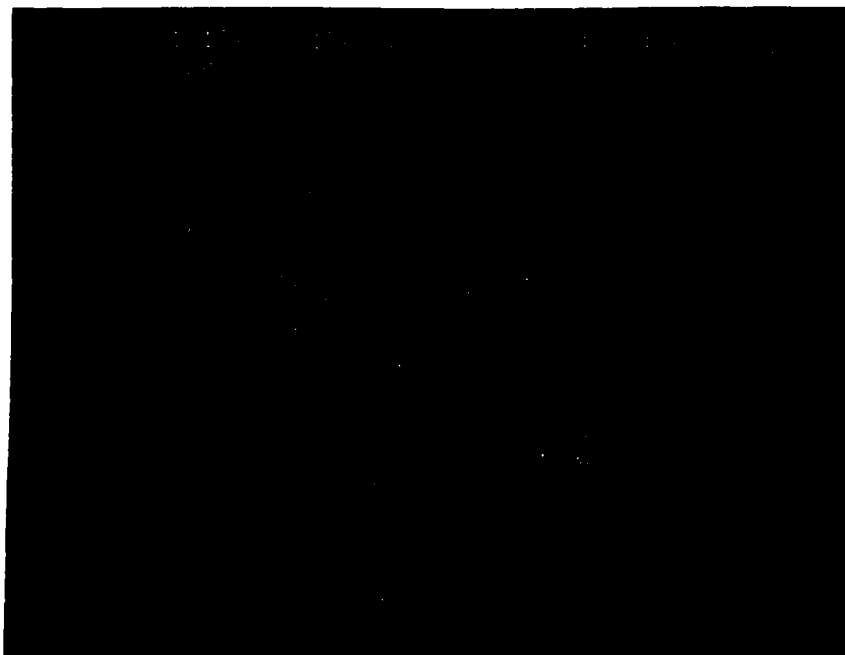


Figure 41b. SEM photograph of *Lactobacillus casei* subspecies *rhamnosus* immobilized onto perlite. (a) The bar represents 20 μm at a 1340x magnification. (b) The bar represents 500 μm at a 52.9x magnification. The cultures were three weeks old.

(a)



(b)

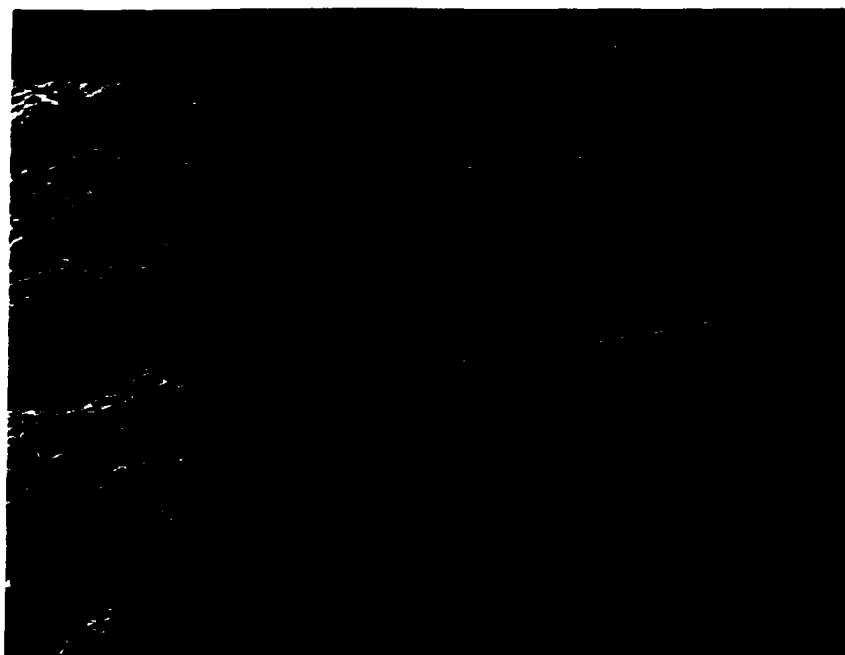


Figure 41c. SEM photograph of *Lactobacillus casei* subspecies *rhamnosus* immobilized onto polystyrene foam. (a) The bar represents 20 μm at a 1130x magnification. (b) The bar represents 500 μm at a 52.8x magnification. The cultures were three weeks old.

(a)



(b)



Figure 41d. SEM photograph of *Lactobacillus casei* subspecies *rhamnosus* immobilized onto polyurethane foam. (a) The bar represents 20 μm at a 1100x magnification. (b) The bar represents 1000 μm at a 49.2x magnification. The cultures were three weeks old.

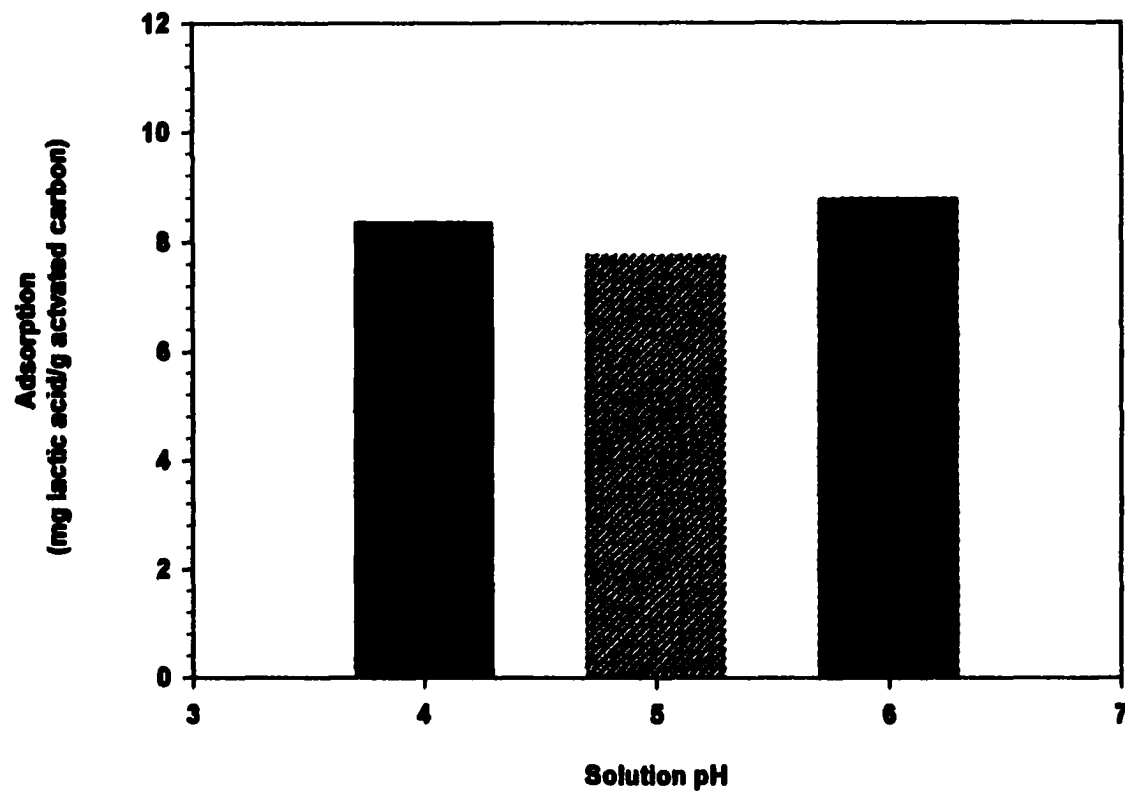


Figure 42. Adsorption of L-(+) lactic acid by granular activated carbon at various pH values. These experiments were conducted in duplicate in sealed tubes at 40°C. The starting L-(+) lactic acid concentration was 4.5 g/l. The sample size was 2 ml. The three pH's selected those are of fermentation broths used in immobilized cultures in this study. The shown values are the average values of duplicated experiments. The range values are 1.4, 2.6 and 0.8 for solution pH's 4, 5 and 6, respectively.

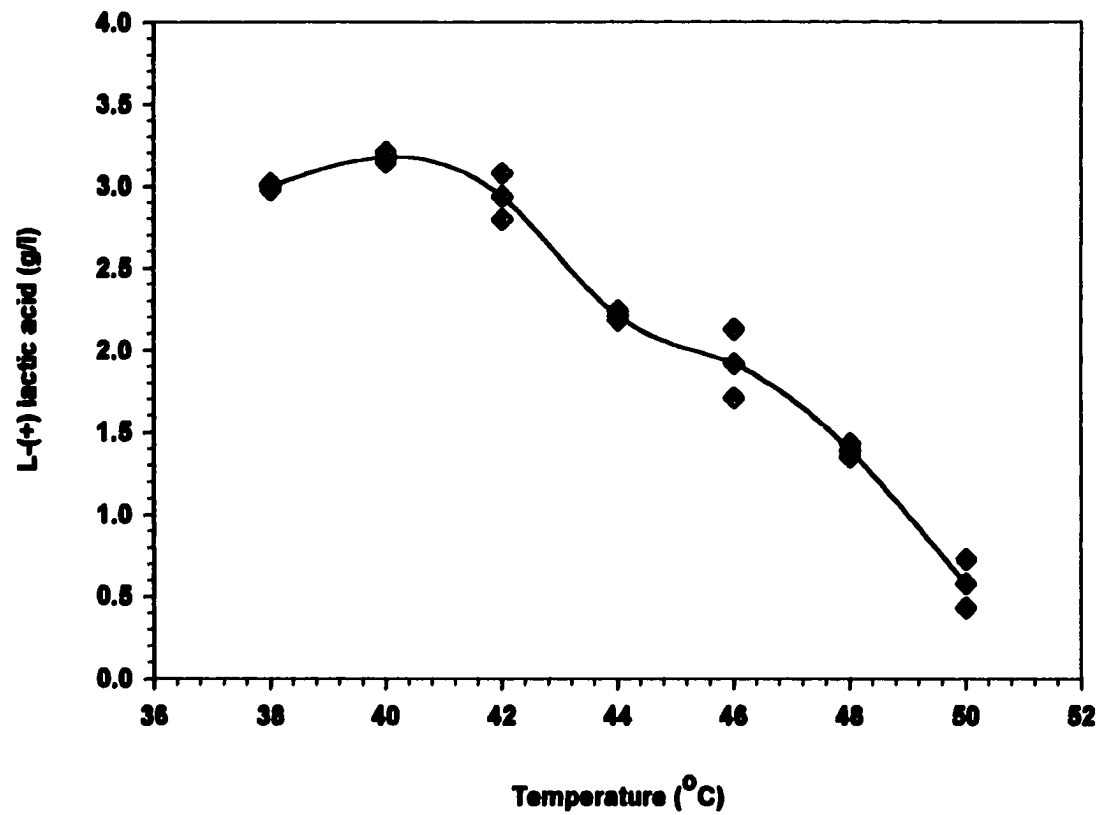


Figure 43. Effect of temperature on L-(+) lactic acid production by immobilized *L. casei* subsp. *rhamnosus*. The bacteria were immobilized onto granular activated carbon and packed in a 10 ml column. Molasses (8% Brix, pH 5.5) was fed at a dilution rate of 1.1/hr. The range bars show the lowest and highest values of duplicated experiments.

acid was produced. Increasing the temperature from 42 up to 50°C also resulted in lower lactic acid production.

The most lactic acid was produced when the feed pH was between 5.5 and 6.0. At a dilution rate of 0.2/hr, 14 ± 1 g/l of lactic acid was produced when the feed pH was 5.5. Decreasing the feed to pH 5 or 4, lactic acid production decreased 30% or 64% , respectively. Increasing the feed pH to 6.5, 52% less lactic acid was produced than when the feed pH was 5.5 (Fig. 44). When the dilution rate was increased to 0.4/hr, 7 ± 1 g/l of lactic acid was produced at a feed pH 5.5. Decreasing or increasing feed pH's to pH 4, 5 or 6.5 resulted in 49, 15 or 25 % less lactic acid production, respectively. The effluent pH's were less acid when the initial pH's were more alkaline . The optimal initial feed pH for immobilized cell cultures was the same as for batch cultures, pH 5.5. This value was used throughout the rest of the study.

At a dilution rate of 0.2/hr, 14 ± 1 g/l of lactic acid was produced. Increasing dilution rates to 0.4, 0.76, 1.1, 1.8, 2.4 or 3.1/hr resulted in lower lactic acid production, 7.2, 4.2, 3.2, 2.3, 2 or 1.6 g/l, respectively. Lower dilution rates gave higher L- (+) lactic acid concentrations, but reduced productivity, indicating that a 10 ml column system was too small for the feed rate (Fig. 45). Residual fermentable sugars were observed. From 8% Brix molasses media, glucose was completely used at a dilution rate of 1.1/hr, while trace of fructose was monitored. At slow dilution rates of 0.2 or 0.4/hr, glucose and fructose were observed in between 1 and 4 g/l. This is because at a slow dilution rate, the sucrose in the fermentation broth was cleaved by the reduced pH. Sucrose was mainly residual in all dilution rates (Fig. 46). At a dilution rate of 0.2/hr, 5.8 g/l of L- (+) lactic acid was produced from 4% Brix molasses (sugar concentration of 33 g/l). Higher molasses concentrations increased lactic acid production at this dilution rate; 14 ± 1 , 13 ± 2 , 12 ± 3 and 8 ± 1 g/l of L- (+) lactic acid produced from 8, 10, 13 and 18% Brix, respectively. At a dilution rate of 0.4/hr, lactic acid production was between 7 to 11 g/l for all molasses concentration, except for the 18% Brix, which produced only 6 ± 1 g/l of lactic acid (Fig. 47).

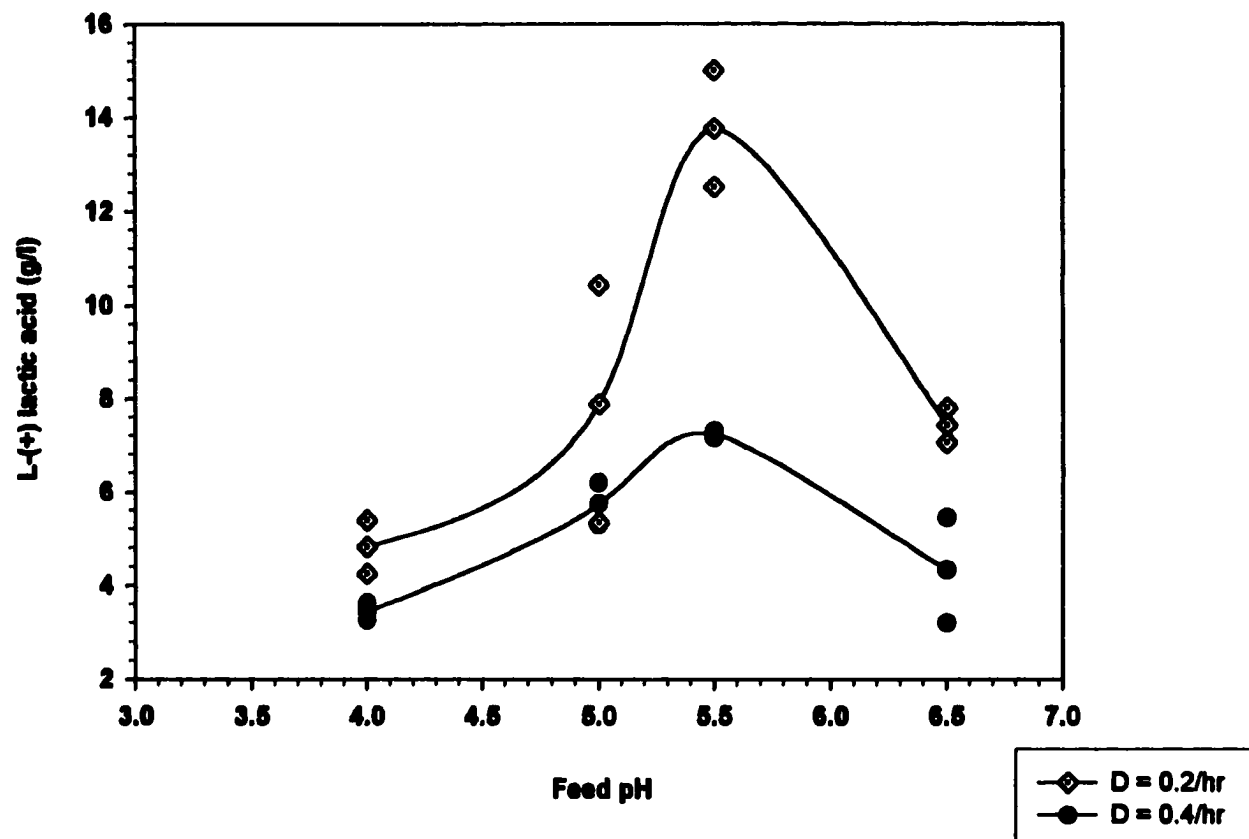


Figure 44. Effect of feed pH on L- (+) lactic acid production from immobilized *L. casei* subsp. *rhamnosus*. The bacteria were immobilized onto granular activated carbon in a 10 ml column. Molasses, 8% Brix (50g/l fermentable sugars), were fed at dilution rates of 0.2 and 0.4 /hr. Temperature was 40°C. The range bars show the lowest and highest values of duplicated experiments.

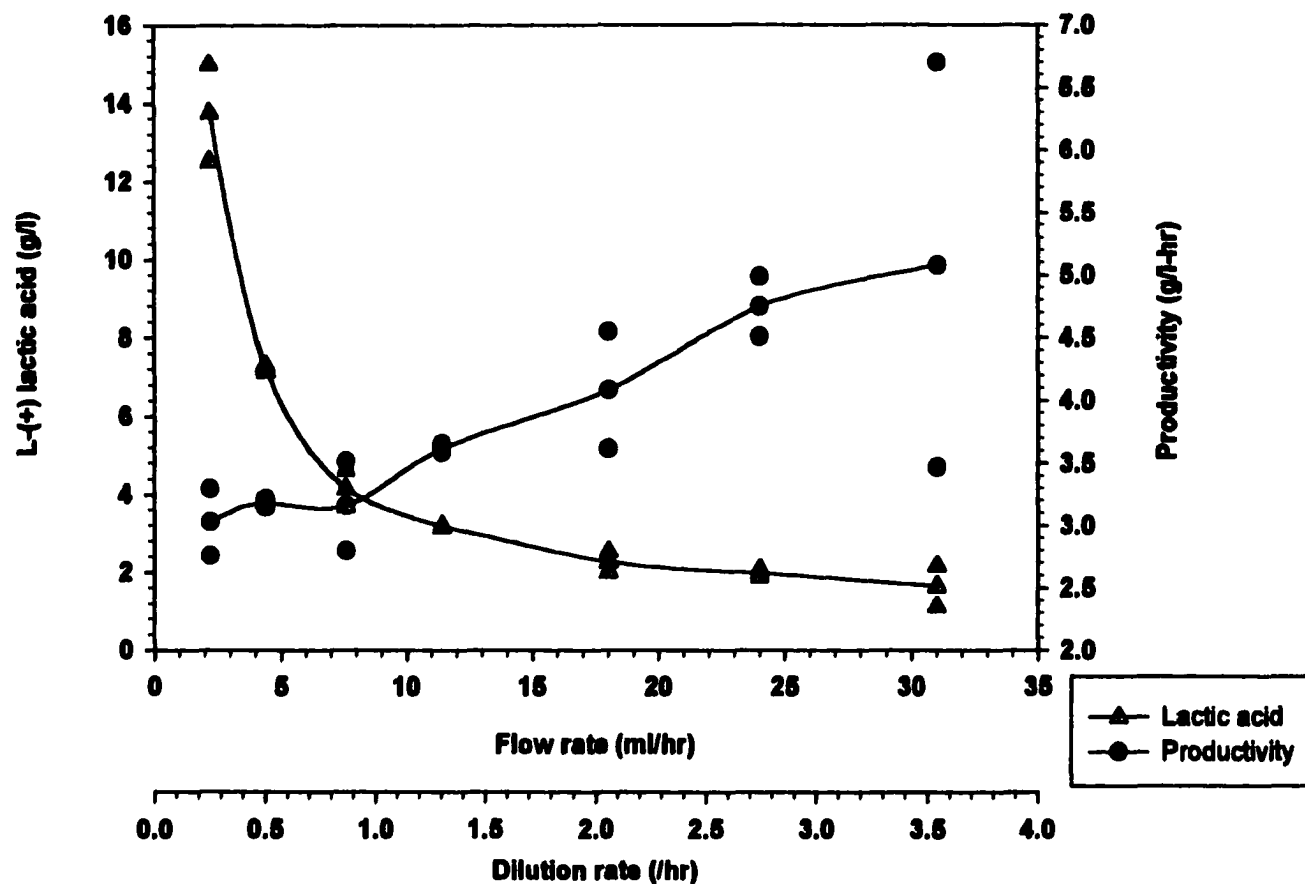


Figure 45. Effect of dilution rate on L-(+) lactic acid production and productivity by immobilized *L. casei* subsp. *rhamnosus*. The experiments were conducted in 10 ml bioreactors. Molasses media, 8% Brix, was adjusted to pH 5.5 prior to use. Temperature was 40°C. The column was allowed to stabilize at each flow rate for 4 days prior to measurement. The range bars show the lowest and highest values of duplicated experiments.

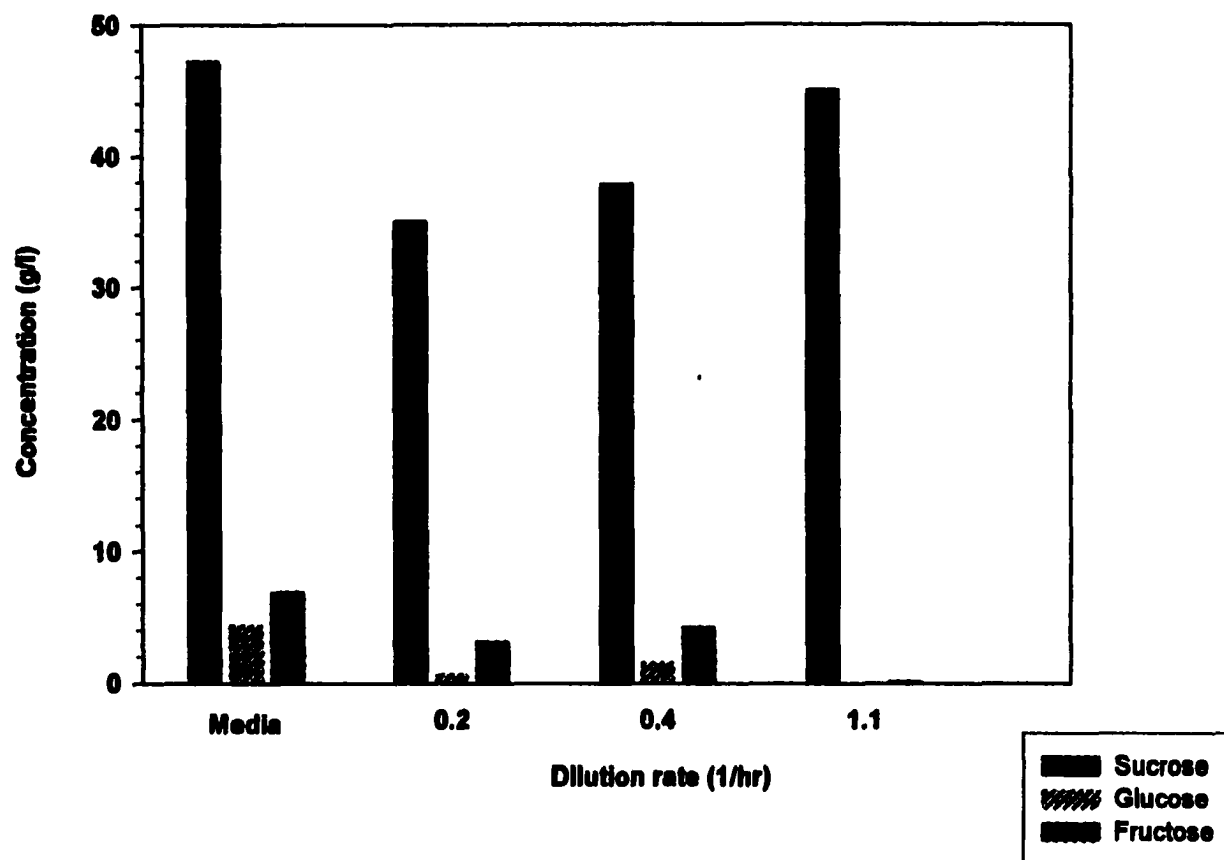


Figure 46. Residual fermentable sugars from immobilized cell fermentation of lactic acid production by *L. casei* subsp. *rhamnosus*. These experiments were conducted in 10 ml bioreactors. Molasses media, 8% Brix, was adjusted to pH 5.5. Temperature was 40°C. Fermentable sugars refer to sucrose, glucose and fructose. The shown values are the average values of duplicated experiments. The range values are in between 0.5 and 1.

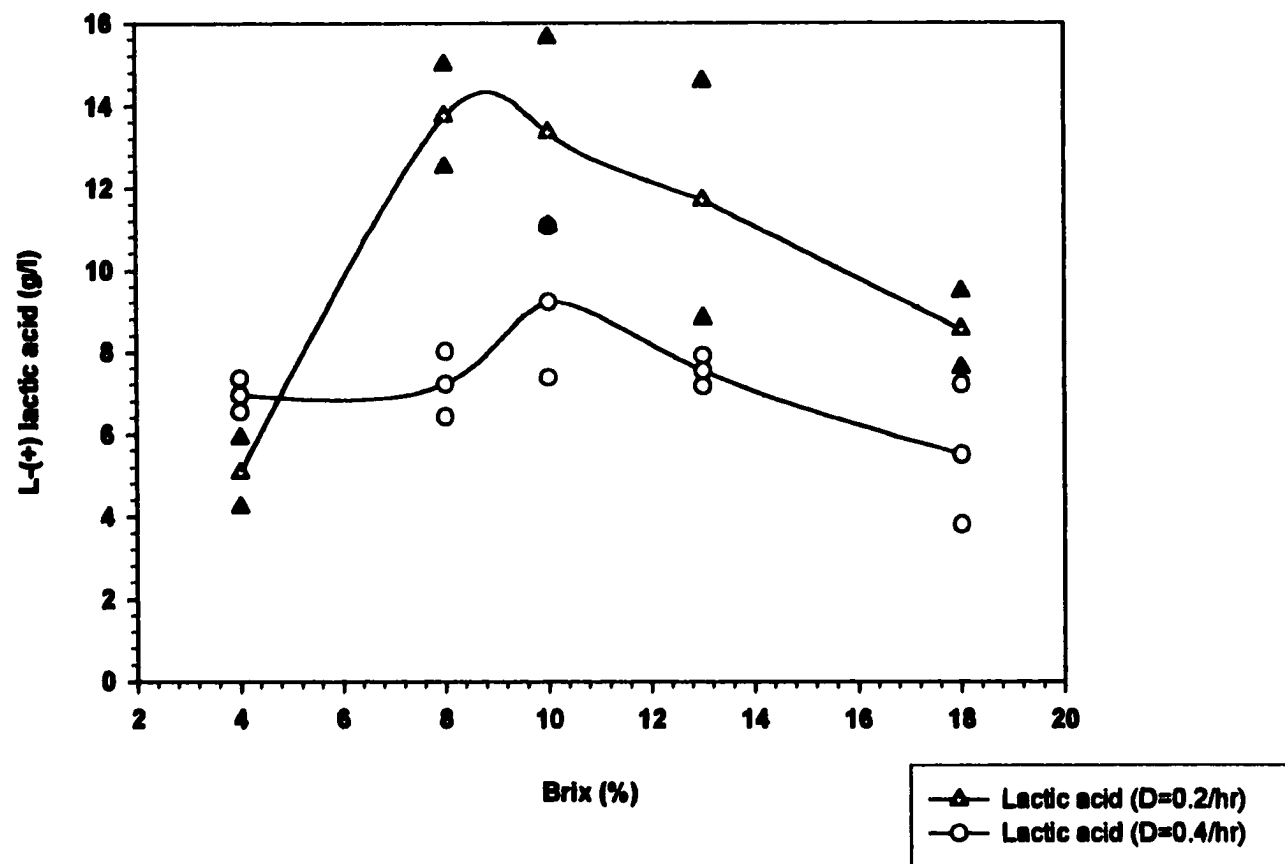


Figure 47. Effect of molasses concentration on L-(+) lactic acid production by immobilized *L. casei* subsp. *rhamnosus*. These experiments were conducted in 10 ml bioreactors, 40°C. Molasses, pH 5.5, fed continuously at dilution rates of 0.2 and 0.4/hr. The fermentations were monitored everyday for 4 days for each condition. The range bars show the lowest and highest values of duplicated experiments.

Molasses, 8% Brix, containing 50 g/l of fermentable sugars, pH 5.5, was used as the feed stock for monitoring the effect of nutrient supplementation on continuously fed immobilized cultures. At a dilution rate of 1.1/hr, yeast extract, 0.2% w/v, produced an increase in L- (+) lactic acid by 29% over control, while 0.4 and 0.8% yeast extract gave 81 and 68% increase over control, respectively. Corn steep liquor, 0.4 and 0.8% (w/v), gave 28 and 19% increase over control, respectively. Ammonium phosphate (dibasic) did not increase lactic acid production while active invertase (1%) increased lactic acid production by 31% (Table 10). When the dilution rate decreased to 0.4/hr, molasses (8% Brix, without nutrient supplementation) produced 7.2 ± 0.1 g/l of lactic acid with a productivity of 3.2 g/l-hr. At this dilution rate, 1% invertase treated molasses gave 7.2 ± 0.6 g/l lactic acid. Similar results were observed at a dilution rate of 0.2/hr. Molasses without nutrient supplementation produced 13.8 ± 1.2 g/l L-(+) lactic acid. When 1% invertase supplemented molasses was fed to a biocolumn, 11.8 ± 1.65 g/l of lactic acid was produced. Invert molasses supplemented with 0.2 and 0.4% yeast extract produced 12.6 ± 1.8 and 12.8 ± 0.7 g/l of lactic acid, respectively (Table 11).

Lactic acid production is an end-product inhibited process. In batch culture, L- (+) lactic acid inhibited *L. casei* subsp. *rharnosus* at a K_i of 355 mM (32 g/l). For immobilized cultures, L- (+) lactic acid was normally produced to 35.5 mM (3.2 g/l) at a dilution rate of 1.1/hr. When 60 mM (5.4 g/l) of L- (+) lactic acid was added, production decreased to 22 mM, and to 8 mM with 140 mM (12.6 g/l) added lactic acid. When 220 mM (20 g/l) L- (+) lactic acid was added to the feed, 7 mM L- (+) lactic acid was produced (Fig. 48). The inhibitory constant (K_i) of L-(+) lactic acid on immobilized culture was not determined as the relationship between added L-(+) lactic acid and lactic acid production was not linear.

As in batch culture, sodium azide decreased lactic acid production. Without sodium azide, 153 ± 13.3 mM (13.8 ± 1.2 g/l) of lactic acid was produced from 8% Brix molasses at a dilution rate of 0.2/hr. When 2 or 8 mM sodium azide was added, L-(+) lactic acid was decreased to 80 or 40 mM, which were a 51 and 76% decrease, respectively (Fig. 49).

Table 10. Effect of nutrient supplementation on L- (+) lactic acid production by immobilized *Lactobacillus casei* subspecies *rhannosus*.

| Supplement | L- (+) lactic acid produced (g/l) | Productivity (g/l-hr) | Increase in lactic acid production over control (%) |
|-----------------------------------|--------------------------------------|--------------------------|--|
| None | 3 | 3.6±0.03 | 0 |
| 0.2% Yeast extract | 4±0.5 | 4.7±0.6 | 29 |
| 0.4% Yeast extract | 6±0.8 | 6.6±0.9 | 81 |
| 0.8% Yeast extract | 5±0.8 | 6.1±0.9 | 68 |
| 0.4% Corn steep liquor | 4±0.2 | 4.7±0.2 | 28 |
| 0.8% Corn steep liquor | 4±0.2 | 4.3±0.2 | 19 |
| 0.2% Ammonium phosphate (dibasic) | 3±0.2 | 3.2±0.2 | 0 |
| 0.4% Ammonium phosphate (dibasic) | 3.2±0.5 | 3.2±0.5 | 0 |
| 1% Invertase (active) | 4.2±0.6 | 4.8±0.7 | 31 |

The experiments were conducted in 10 ml bioreactors. Molasses media, 8% Brix, were supplemented with desired nutrients and pH was adjusted to pH 5.5. Media were fed at a dilution rate of 1.1 hr^{-1} (11.4 ml/hr flow rate). Temperature was 40°C. Shown values are range values of two repetitions.

Table 11. Effect of inversion of molasses on L- (+) lactic acid production by immobilized *L. casei* subsp. *rhamnosus*.

| Supplement | D = 0.2/hr | | D = 0.4/hr | |
|-------------------------|--------------------------|---|--------------------------|---|
| | L- (+) lactic acid (g/l) | Increase in production over control (%) | L- (+) lactic acid (g/l) | Increase in production over control (%) |
| A. Not inverted | | | | |
| 1. Not supplemented | 14±1 | 0 | 7±0.1 | 0 |
| 2. + 0.2% Yeast extract | 11±1 | 0 | 6±1 | 0 |
| B. Inverted | | | | |
| 1. Not supplemented | 12±1.6 | 0 | 7±0.6 | 0 |
| 2. + 0.2% Yeast extract | 12±2 | 0 | 7±1 | 3 |
| 3. + 0.4% Yeast extract | 12±1 | 0 | NC | NC |

The experiments were conducted in 10 ml bioreactors without pH control. Sucrose in molasses media, 8% Brix and pH 5.5, was inverted into glucose and fructose by an invertase enzyme prior to use. The media were or were not supplemented. Temperature was 40°C. NC refers to not conducted. Production from non-supplemented molasses was used as a control. Shown values are means and range values of two repetitions.

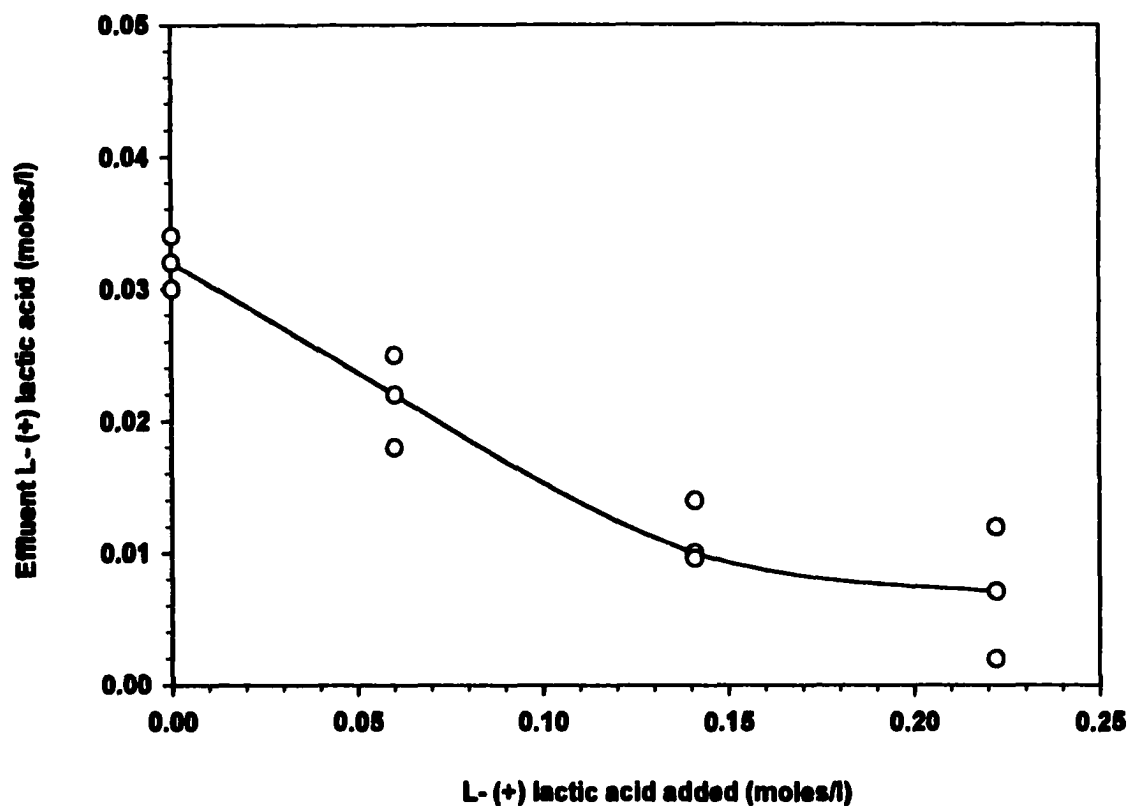


Figure 48. Effect of external L-(+) lactic acid on lactic acid production by immobilized *L. casei* subsp. *rhamnosus*. L-(+) lactic acid, 0, 0.06, 0.13 and 0.22 moles/l, in molasses media (8% Brix) were fed at constant flow rates of 11.4 ml/hr to immobilized cell reactors (10 ml in size). The media were adjusted to pH 5.5 prior to use. Culture temperature was 40°C. Each column was stabilized for 4 days prior to measurement. The range bars show the lowest and highest values of two repetitions.

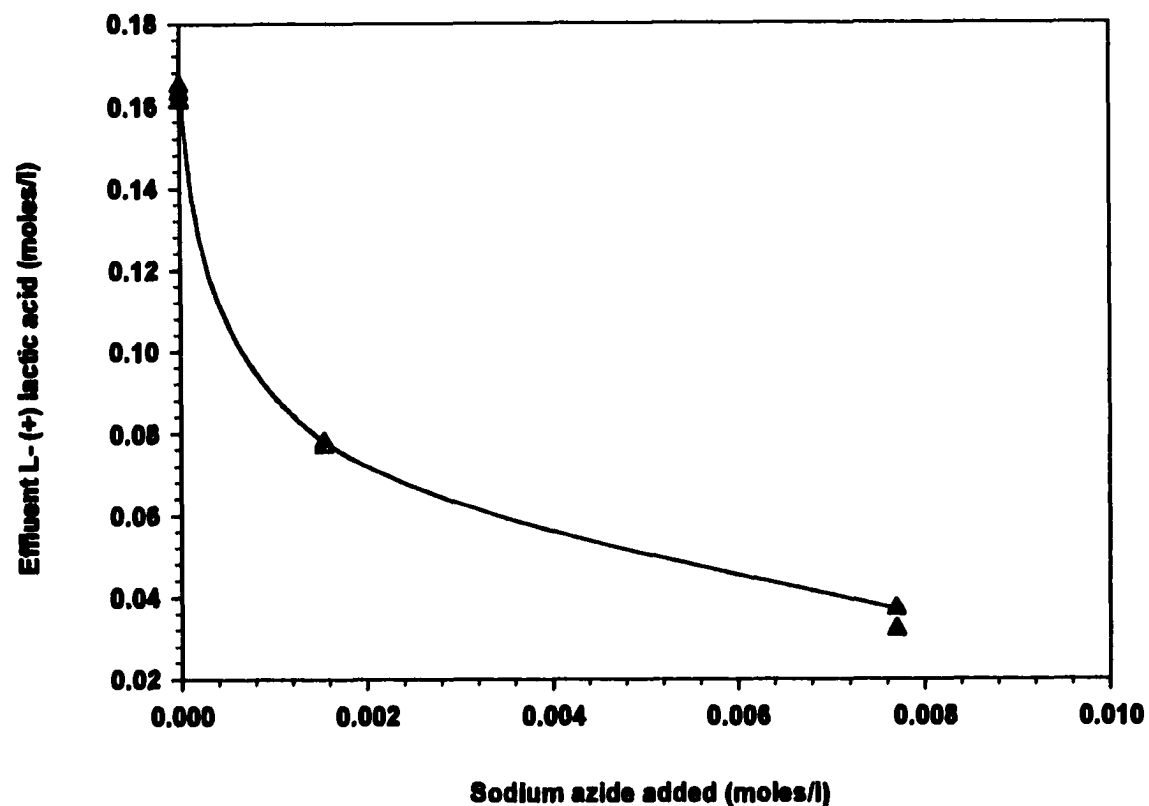


Figure 49. Effect of sodium azide on L- (+) lactic acid production by immobilized *L. casei* subsp. *rhannosus*. These experiments were conducted in 10 ml immobilized cell reactors. Sodium azide, 0, 1.54×10^{-3} and 7.69×10^{-3} moles/l, in molasses media, 8% Brix, were fed to the column at a constant flow rate of 2.2 ml/hr (dilution rate of 0.2/hr). Media were adjusted to pH 5.5 prior to use. Temperature was 40°C. The range bars show the lowest and highest values of two repetitions.

Summary of immobilized cell fermentation for lactic acid production from 10 ml bioreactors is shown in Table 12.

C.4 Immobilized cell chemostat (400 ml)

In a larger immobilized cell column, yeast extract (0.4% w/v) was added to molasses medium, 8% Brix, pH 5.5, and fed to the column at the dilution rate of 0.04/hr (15 ml/hr flow rate), without pH control. The culture pH was dropped from 5.5 to 3.8 after 81 hours of operation. L- (+) lactic acid, 14.8 ± 0.6 g/l, was produced with a productivity of 0.59 g/l-hr.

When the column was controlled at pH 5.5, cell numbers were higher than without pH control. L-(+) lactic acid, 20 ± 1 g/l, was produced with a productivity of 0.8 g/l-hr. The efficiency of pH control using media recycling was poor because the culture broth did not rapidly mix throughout the reactor, resulting in pH gradients. The pH at the bottom of the reactor was lower than at the middle or the top of the column.

When molasses concentration was increased to 13% Brix, (other factors were not changed), L- (+) lactic acid, 31 ± 4 g/l, was produced with a productivity of 1.2 g/l-hr without pH control. When molasses concentration increased from 8 to 13% Brix, more lactic acid was produced, and numbers of cells releasing from the fermentor increased (Table 13). Contamination was observed after 3 weeks of operation.

D. L- (+) lactic acid production (pilot plant)

D.1 Without pH control. The working volume of the pilot plant was 5.36 liters. Molasses media containing 3.4 g/l fermentable substrate, 0.8% Brix, containing 0.005% sodium azide to reduce contamination was used. At a dilution rate of 0.01/hr, the first segment of the pilot column did 78% of the conversion (0.78 ± 1.2 g/l) of sugar to lactic acid while 15% (0.15 ± 0.1 g/l) and 7% (0.07 ± 0.06 g/l) were obtained from segments 2 and 3, respectively. The culture pH dropped rapidly across the first segment (2.3 pH points) and either then dropped slowly, 0.2 points, or was stable over the next two segments (Fig. 50 and 51).

Table 12. Summary of lactic acid production from blackstrap molasses by immobilized *L. casei* subsp. *rharnosus* from 10 ml bioreactors.

| Condition | Result |
|-----------------------------------|--------|
| Temperature (°C) | 40 |
| Media pH | 5.5 |
| Dilution rate (1/hr) | 0.2 |
| Molasses concentration (% Brix) | 8 |
| Nutrient supplementation | None |
| Lactic acid concentration (g/l) | 14 ± 1 |
| Inhibited by sodium azide | Yes |
| Inhibited by external lactic acid | Yes |

Table 13. L- (+) lactic acid production by *Lactobacillus casei* subspecies *rhamnosus* immobilized onto activated carbon in a 400 ml column.

| Condition | Molasses (%) Brix | L-(+) lactic acid (g/l) | Cell count (10 ⁶ CFU/ml) | Productivity (g/l-hr) |
|------------------------------|-------------------|----------------------------|--|-----------------------|
| 1. <u>Without pH control</u> | 8 | 15±0.6 | 182 | 0.6 |
| 2. <u>With pH control</u> | 8 | 20±1.7 | 1750 | 0.8 |
| | 13 | 31±4.3 | 2500 | 1.2 |

The temperature was 40°C. For pH control, NaOH, 5% w/v, was used. The molasses media (untreated with invertase or heat) were adjust to pH 5.5 and supplemented with yeast extract, 0.4% w/v prior to use. Molasses were fed at a dilution rate of 0.04/hr (15 ml/hr flow rate). Values shown are mean values of two repetitions.

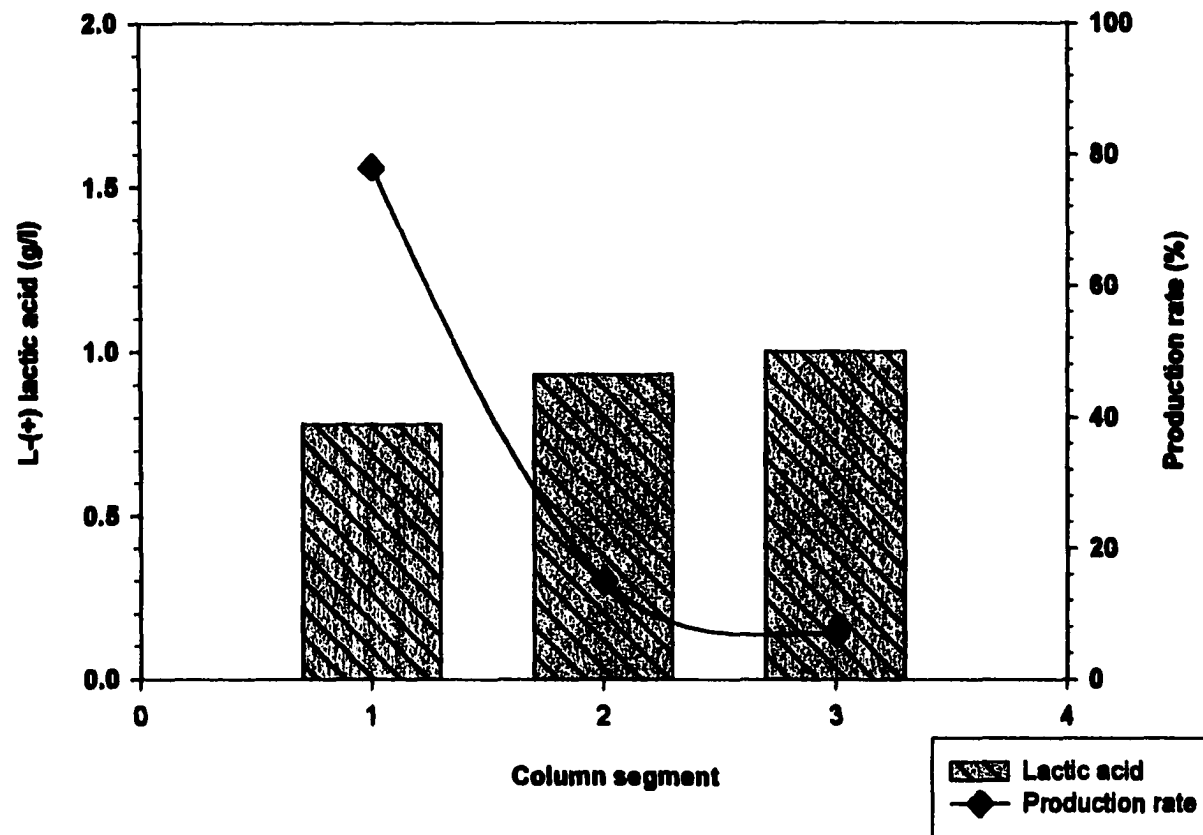


Figure 50. Segment characteristics of the pilot plant. These experiments were conducted without pH control. Molasses media contained 3.4 g/l fermentable sugars (sum of sucrose, glucose and fructose). The column was fed at a dilution rate 0.01/hr (a flow rate of 60 ml/hr). Temperature was 42°C. The plant was monitored for 4 days. The shown values are the average of two repetitions. The range value is 0.1 for all segments.

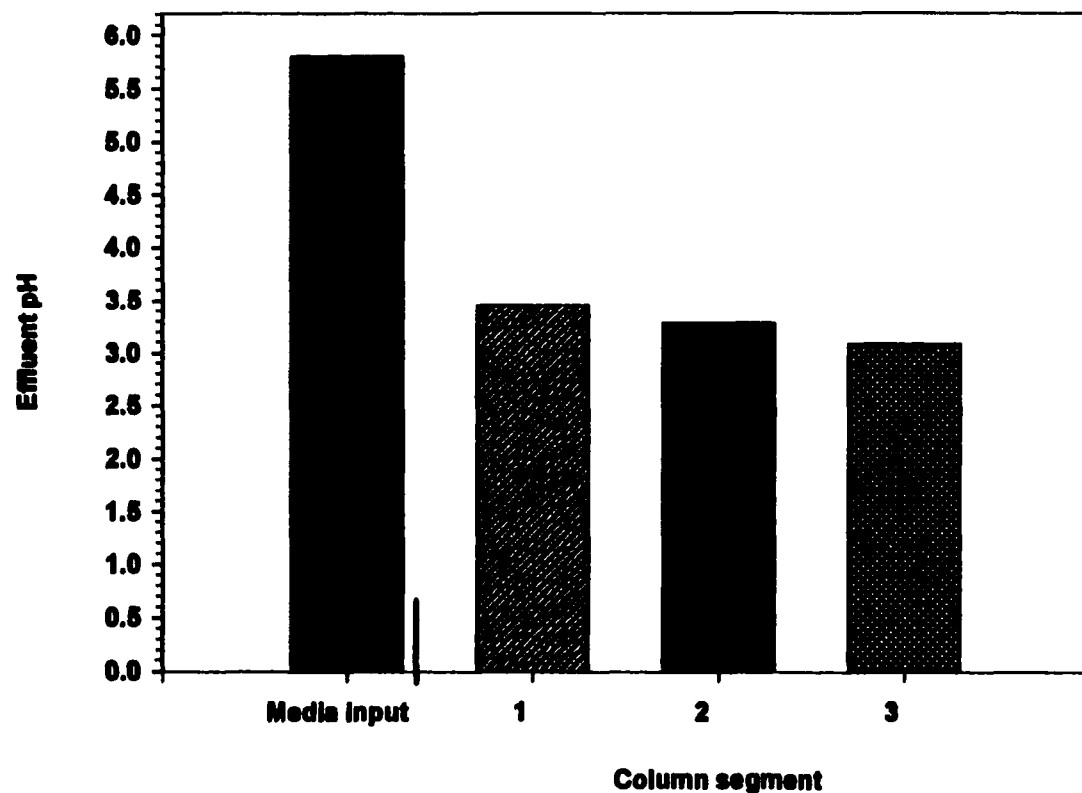


Figure 51. Segment characteristics of the pilot plant. These experiments were conducted without pH control. Molasses media contained 3.4 g/l fermentable sugars (sum of sucrose, glucose and fructose). The column was fed at a dilution rate 0.011/hr (a flow rate of 60 ml/hr). Temperature was 42°C. The bioreactor was monitored for 4 days. The shown values are the average values of two repetitions. The range values for all data are in between 0.1 and 0.2.

Because of static flow in the column, there was both a sugar and lactic acid gradient from top to bottom (Table 14). At a dilution rate of 0.1/hr, samples were taken from different levels and analyzed for sugar and lactic acid content. At the bottom, more sugar (17 g/l) and lactic acid (2.4 g/l) was found than in the middle or top of the column. There was also a temperature gradient across the system, 45°C at the bottom, 42°C in the middle and 39°C at the top.

D.2 With pH control. NaOH, 5%, was pumped through spargers which were installed in the pilot plant to control pH within the pilot plant. The column was controlled at pH 4 to reduce contamination and was fed with media containing 0.005% sodium azide. Without sodium azide, significant contamination by Gram-negative bacteria and yeasts was observed. However, this pH control method was not completely successful. A mixer had to be installed in between the column segment 1 and 2. Sodium hydroxide was used to control the pH between the internal segments 2 and 3. At molasses concentrations of 2, 4, 8 and 13 % Brix (8, 19, 38 and 68 g/l fermentable sugars, respectively) and a dilution rate of 0.06 /hr (300 ml/hr flow rate), 0.8, 1.3, 3.3 and 5.0 g/l L- (+) lactic acid was produced with productivity values of 0.05, 0.08, 0.20 and 0.30 g/l-hr, respectively. The molar conversions were 0.31, 0.24, 0.55 and 0.52 moles lactic acid/moles hexose utilized for 2, 4, 8 and 13% Brix, respectively. The residual fermentable sugars 2.6, 8.1, 25.8 and 49.3 g/l for 2, 4, 8 and 13% Brix molasses media, respectively (Fig. 52).

II. Purification of Lactic Acid

Purified L- (+) lactic acid absorbed onto an anionic resin, Amberlite IRA-400, at the capacity of at least 29 mg L- (+) lactic acid/g resin. Using spent fermentation broth, 34 mg L- (+) lactic acid was absorbed per gram of the resin. The absorbed lactic acid could be eluted by using 2N HCl. The adsorption, before elution, was at pH 8. The recovery of lactic acid was 95% for purified lactic acid and 56% for spent fermentation broth (Fig. 53 and 54). From spent fermentation broth, the lactic acid was not completely recovered from the resin, regardless of an increase in strength in the hydrochloric acid.

Table 14. Pilot plant profile.

| Column segment | Sample collecting level (distance from the bottom, inches) | Fermentable sugars(g/l) | L-(+) lactic acid (g/l) |
|----------------|---|-------------------------|-------------------------|
| 1 | 0 | 25±1.6 | 2.6±0.8 |
| | 2 | 14±0.7 | 1.2±0.2 |
| | 4 | 7.6±0.5 | 0.07±0.05 |
| 2 | 0 | 26±2.2 | 2.8±0.2 |
| | 2 | 13±0.2 | 1.2±0.3 |
| | 4 | 7.7±0.2 | 0.1±0.05 |
| 3 | output | 7.6±1 | 0.25±0.1 |

Fermentable sugars refer to the sum of sucrose, glucose and fructose. Molasses media containing 9 g/l fermentable sugars was fed at a dilution rate of 0.11/hr. Samples were collected from three levels from the bottom; 0, 2 and 4 inches. Shown values are mean values of two repetitions.

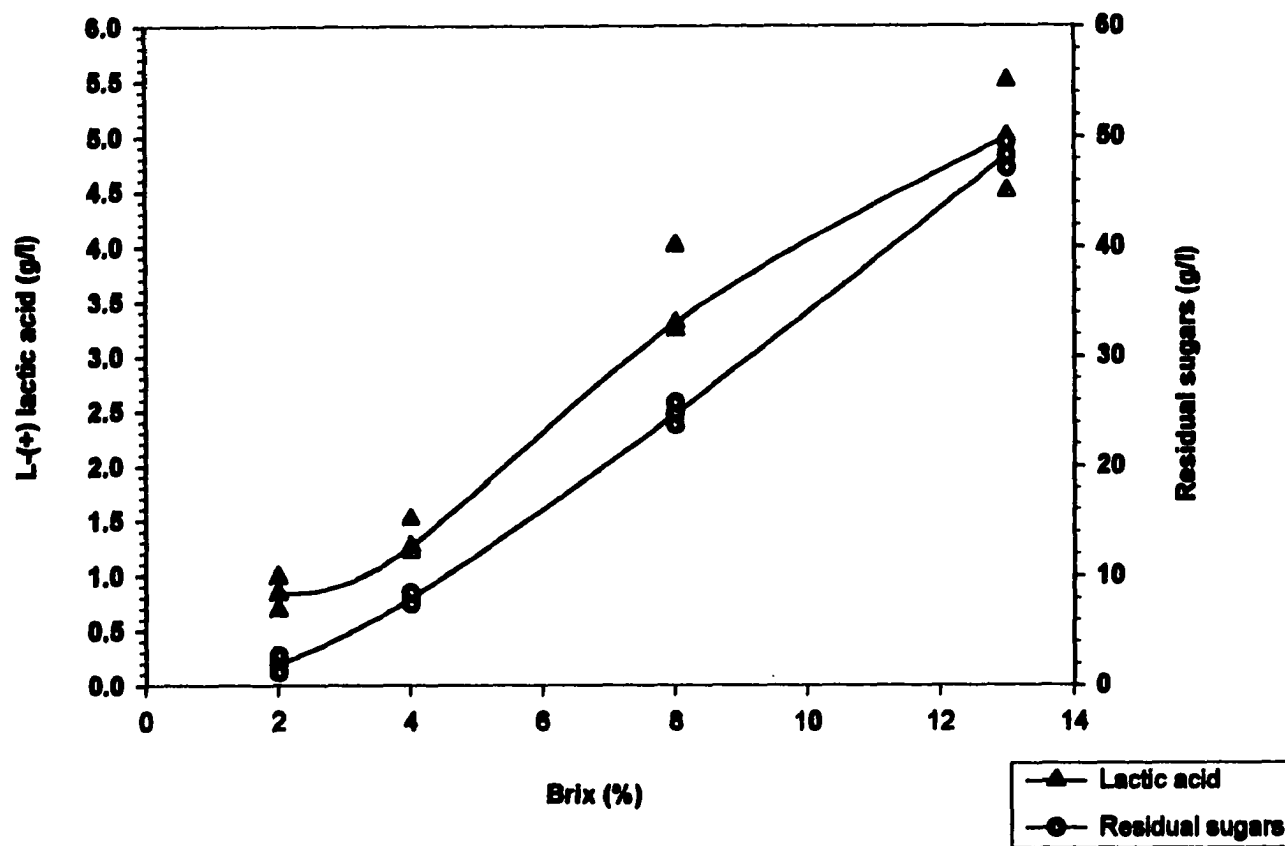


Figure 52. Summary of the pilot unit operation. The plant was fed with media at a dilution rate of 0.06/hr (300 ml/hr flow rate). Fermentable sugars refers to a sum of sucrose, glucose and fructose. Fermentations were controlled at pH 4, 42°C. Samples were collected from the position at the middle of the column of each segment (2 inches from the bottom). Molasses media were supplemented with 0.005% sodium azide. The range bars show the lowest and highest values of duplicated experiments.

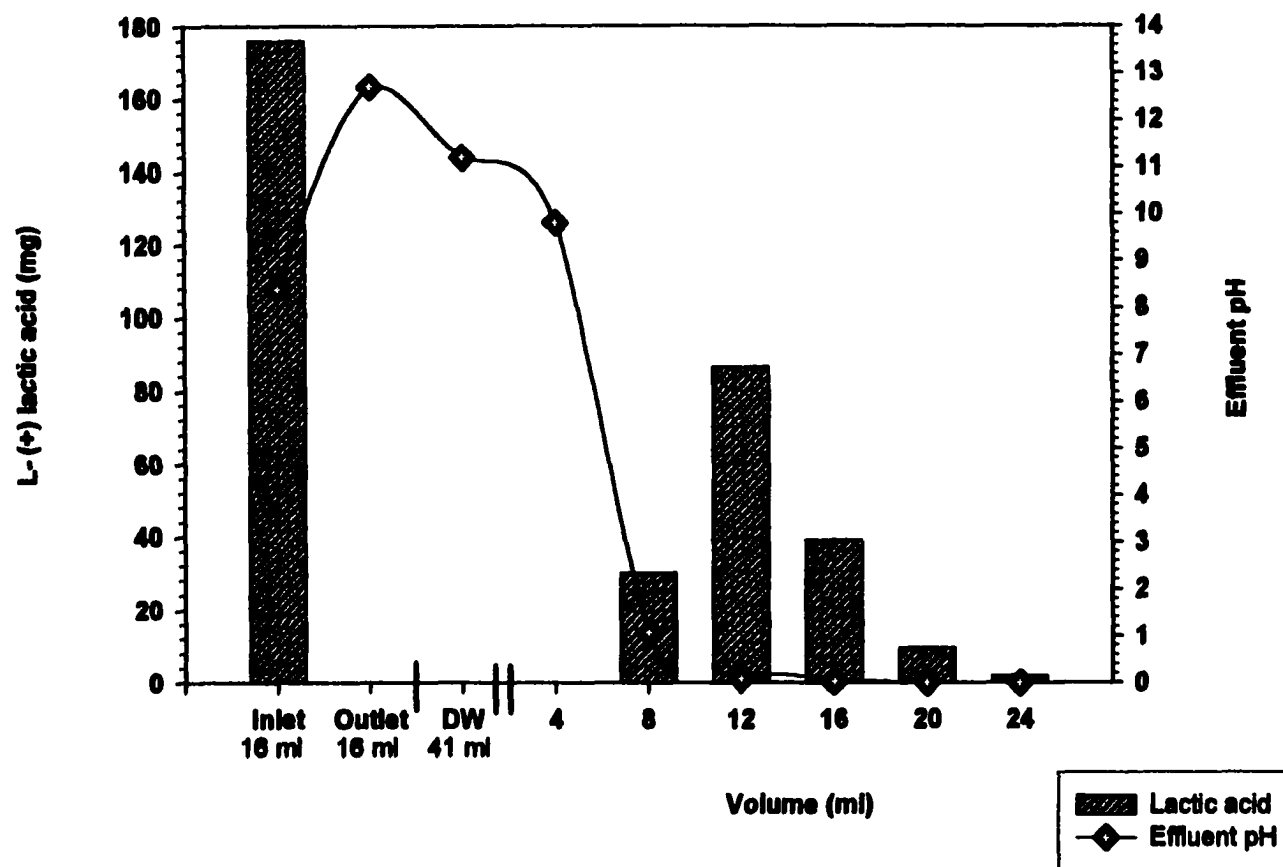


Figure 53. Purification of L-(+) lactic acid using an anionic resin column. Amberlite IRA-400, 6 g, was packed in a 10 ml column. Purified L-(+) lactic acid was fed at a flow rate of 15 ml/hr. The bound acid was then eluted on 4 ml fraction basis with 2N HCl. The adsorption was 29 g lactic acid/g resin. The lactic acid recovery was 95%. Inlet is the solution before being pumped into the resin column. Outlet is the solution after the resin column. DW is distilled water.

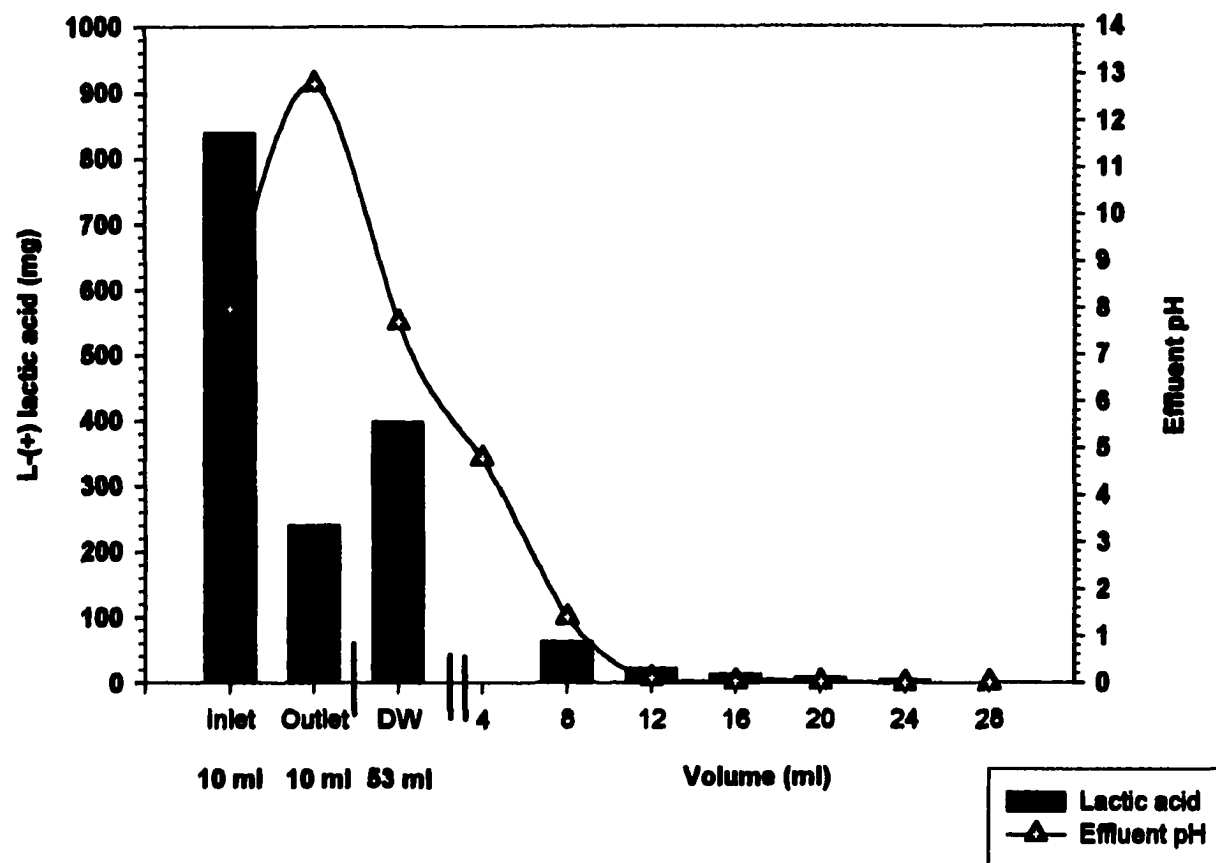


Figure 54. Purification of L-(+) lactic acid from spent fermentation broth by using anionic resin column. Amberlite IRA-400, 6 g, was packed in a 10 ml column. Spent broth was centrifuged to remove any precipitates. The broth was adjusted to pH 8 before passing into the column at a flow rate of 15 ml/hr. The bound acid was eluted with 2 N HCl. The adsorption was 34 mg/g resin. The recovery was 56%. Inlet is the fermentation broth that was pumped into the resin column. Outlet is the broth after the resin column. DW is distilled water.

III. Financial Analysis of Lactic Acid Production

The analysis used only costs of carbohydrate substrate and nutrients. Sugarcane molasses price is \$35/ton, while yeast extract or invertase costs \$7.11 to \$9.35/kg. Other costs of lactic acid production are assumed to be the same as the conventional method.

A. Batch fermentation: The most efficient lactic acid production was from 8% Brix, supplemented with 0.2% (w/v) invertase. L-(+) lactic acid produced was 50 g/l. The unit cost of molasses to produce one kilogram of lactic acid was \$ 0.07. However, when the invertase cost was added (assuming that it has the same price as yeast extract), the production cost jumped up to \$0.35/kg lactic acid. Cost of lactic acid production was mainly due to nutrient supplementation. Yeast extract is currently used in conventional production methods for lactic acid.

B. Continuous, immobilized fermentation: The highest lactic acid yield was obtained at a dilution rate of 0.2/hr, where 14 ± 1 g/l of lactic acid was produced from 100g molasses. Residual fermentable sugars were 39 g/l which had 68% remaining value. If molasses cannot be resold, lactic acid was produced at a carbohydrate cost of \$0.24/kg. However, if molasses in the fermentation broth was resold based on the content of residual sugars, the production cost lower to \$0.075/kg lactic acid. Summary of carbohydrate and nutrient financial analysis is shown in Table 15 and 16.

Table 15. Financial analysis of L-(+) lactic acid production by *L. casei* subsp. *rhannosus*.

| Methods | | Batch fermentation | Immobilized cell fermentation |
|---------|---------------------------------------|--------------------|-------------------------------|
| 1. | Molasses used (g/l) | 100 | 100 |
| | Fermentable sugars(g/l) | 50 | 58 |
| | Cost of molasses used(\$) | 0.0035 | 0.0035 |
| 2. | Yeast extract or invertase used (g/l) | 2 | none |
| | Cost(\$) | 0.014 | none |
| 3. | Lactic acid productivity (g/l-hr) | 1.0 | 2.8 |
| 4. | Lactic acid produced (g/l) | 50 | 14±1 |
| 5. | Production cost (\$/kg) | 0.35 | 0.24 |

The analysis is based on carbohydrates and nutrient supplements cost only. Residual molasses is not resold.

Table 16. Financial analysis of L-(+) lactic acid production by *L. casei* subsp. *rhamnosus*.

| Methods | | Batch fermentation | Immobilized cell fermentation |
|---------|--|--------------------|-------------------------------|
| 1. | Molasses used (g/l) | 100 | 100 |
| | Fermentable sugars(g/l) | 50 | 58 |
| | Cost of molasses used(\$) | 0.0035 | 0.0035 |
| 2. | Residual sugars (g/l) | 0 | 39 |
| | Remaining values (%) | 0 | 68 |
| | Cost of molasses used after reselling (\$) | 0.0035 | 0.0011 |
| 3. | Yeast extract or invertase used (g/l) | 2 | none |
| | Cost(\$) | 0.014 | none |
| 4. | Lactic acid productivity (g/l-hr) | 1.0 | 2.8 |
| 5. | Lactic acid produced (g/l) | 50 | 14±1 |
| 6. | Lactic acid production cost (\$/kg) | 0.35 | 0.075 |

The analysis is based on carbohydrates and supplements cost only. In this case, molasses is resold after lactic acid purification.

DISCUSSION

Sugar is a major component of Louisiana's agricultural sector. Sugarcane has been commercially grown in South Louisiana for over 250 years. Today, the economies of parishes in which sugarcane is grown are still largely based on income derived from sugarcane production and processing. The direct value of this crop, not including value added calculations is in excess of \$300,000,000. Yet, the sugar industry in Louisiana has always been considered a "marginal" industry, as the sugarcane growing belt in this state is on the climatic edge of possible sugarcane producing regions of the world. This means the production of only "immature" sugarcane. The resultant sugar yields per acre are low compared with tropical growing regions. The Louisiana industry has always managed to stay economically competitive, partially through increased technical effort and higher efficiencies, and there is still room for some improvement in this area, but the costs per unit improvement are growing substantially. Currently, the survival of the existing industry in Louisiana is pegged to a federally supported price for sugar of \$0.18-0.20 per pound. Curtailment or reduction in the current price structure will have adverse effects on the domestic sugar industry and the economy of Louisiana.

The keys to the survival of this industry are diversification of output and efficient utilization of existing physical plants. Increased utilization of existing plants would have the greatest effect in Louisiana where the sugar producing plant is operational only three months of the year. Diversification would reduce the overall dependence of a significant sector of this State's economy on a single product (raw sugar). Individual sugar producers in the state have been moving, albeit with halting steps, towards diversification. The size of the Louisiana industry is such that it is not practical for individual factories to directly compete with large well established suppliers of other products (for example in pharmaceuticals or fructose syrups-corn). They must look to small specialty markets to expand their product base or establish new markets for their existing products. Products would be preferentially produced on site with at

least partial utilization of existing facilities. They may be produced year round. Such commodities should offer the Louisiana sugar producer an opportunity for expansion of his product base, freeing him from dependence on a controlled market price. Lactic acid may be such a product.

Lactic acid, the "commodity chemical sleeping giant", is a commodity organic chemical that is used in food and pharmaceuticals. It is also the starting material for polylactic acid, the prime component of the newest and most promising of the biodegradable plastics. This plastic requires an optically pure isomeric form, D or L, for production. L-(+) lactic acid is the preferred starting isomer as its polymers tolerate higher temperatures and humidity. In 1998, approximately 130 million pounds of L-(+) lactic acid was produced with a value of 150 million dollars. The purified isomeric form of lactic acid can be only produced by microbial fermentation. Commercial lactic acid production is by batch fermentation. Continuous cell fermentation has been investigated but not utilized commercially (Severson, 1998). Production of this chemical from molasses could better provide a stable market for molasses, or if done in a sugar mill, provide a new value-added product for this industry. Either way, this can help establish an economic safety net that will ensure the survival of a Louisiana industry while at the same time encouraging development of a biotechnically based industry that can function as a magnet for further investment.

Lactobacillus casei subspecies *rhannosus* produces L-(+) lactic acid in order to compete with other microorganisms, by excreting the acid and reducing the surrounding pH (Axelsson, 1993). *L. casei* subsp. *rhannosus* is a fastidious organism but it was found to grow on diluted blackstrap molasses although with a lag phase that was longer than on synthetic media. The strain immobilized onto a solid support. Four solid supports were tested, and granular activated carbon was found to be best for this purpose. Granular activated carbon provides rough surface with many pores of a size suitable for protection of a population. Scanning electron microscopic pictures of granular activated carbon attached with *L. casei* subsp. *rhannosus* showed that the attached bacteria produce exopolysaccharide, which is

consistent with other reports of exopolysaccharide production by *L. casei* (Mozzi, et al., 1994). Other lactic acid bacteria have also been reported to produce exopolysaccharides. *Lactobacillus casei* CG11 produced exopolysaccharide when grown on glucose (Cerning, et al., 1994), *Lactobacillus* L191 produced exopolysaccharide when grown on a sucrose-rich media (Dykes, et al., 1995), *Streptococcus mutans* and *Streptococcus sobrinus* produce soluble and insoluble glucans, which promote bacterial adherence to smooths dental surfaces and promote dental plaque (Cerning, 1990). The exopolysaccharide produced by *L. casei* subsp. *rhamnosus* may help anchor this bacteria to the support.

Lactobacilli are mesophiles. They are normal flora of human and animal gastrointestinal tracts, and grow between 37 and 42°C (Jin, et al., 1996). *L. casei* subsp. *rhamnosus* showed its best growth and lactic acid production at 40±2°C. This species has been studied by other groups, who reported optimal growth and lactic acid production at either 37 or 42°C (Ho, et al., 1997, Hujanen, et al., 1996 and Senthuran, et al., 1997). The ATCC 10863 strain had a maximum growth temperature of 42°C (Yoo, et al., 1997). The preference for high temperature of the bacterium is an advantage for lactic acid production as it reduces competitive microorganisms.

L. casei subspecies *rhamnosus* grew best on molasses at a pH between 5.5, which was similar to other reports (Ho, et al., 1997 and Senthuran, et al., 1997). In batch culture, this organism did not grow at pH values below 3.5. *L. casei* subsp. *rhamnosus* grew slowly at pH values above 5.5. In molasses media, the highest growth rate was at pH 5.5. At pH 5.5, the bacteria grew faster and produced more cell mass when grown on MRS than on molasses media. Immobilized cultures at pH 5.5 produced the most lactic acid. Lactobacilli have been reported to withstand low pH values, in fact, they tolerate the lowest pH's among the lactic acid bacteria. Generally, lactic acid bacteria maintain cytoplasmic pH values slightly higher than the environmental pH. When their cytoplasmic pH is lower than the threshold pH, metabolic pathways that are acid sensitive, particularly glycolysis, cease and the cells die. Lactobacilli maintain a cytoplasmic pH of 4.4 when the external pH is 3.5 (Bender, et al., 1987).

Lactobacillus casei ATCC 4646 requires a pH of approximately 3.2 for glycolysis. *S. mutans* GS-5 has the minimum pH of 4.0 while other dental plaque members, such as *Actinomyces viscosus* OMZ105E have a minimum pH above 5.0. The acid tolerance of lactic acid bacteria is a function of proton-translocating ATPases. Isolated membranes of *L. casei* ATCC 4646 produce approximately 3.29 units/mg protein as ATPase with an optimum activity at pH 5, whereas *A. viscosus* produces about 0.06 units/mg protein as ATPase with an optimum activity at pH 7. Cytoplasmic pH maintenance is regulated by a H⁺-coupled ion transport system (Booth, 1985). Bacteria that produce acids as end-products transport weak acids, but not lactic acid, by diffusion through the cell membranes. The cell membrane is permeable to the lipophilic, unionized forms of the acid and is impermeable to hydrophilic, charged forms. Lactate is transported by a membrane carrier and an electrogenic porter of H⁺/lactate (Kashket, 1987). Protons, which are electrogenic, are transported across the cytoplasmic membrane collapsing pH gradient (Kashket, 1987). At pH higher than 5.5, the bacterial enzymes could not function due to the higher than normal of cytoplasmic pH values. It has been reported that L-LDH of *L. casei* has the pH optimum of less than 5.5 (Mary, *et al.*, 1980, and Russel and Hino, 1985). *L. casei* subsp. *rhamnosus*, did not grow well at a pH lower than pH 5.5, probably due to its inability to excrete acid out of the cell to maintain a higher cytoplasmic pH than its surrounding pH.

High external lactic acid concentrations affect both bacterial growth and lactic acid synthesis (Monteagudo, *et al.*, 1997). *S. faecalis* AHU 1256, another lactic acid bacteria, had a growth inhibition constant (K_i) of 9.5 g/l lactic acid (Ohara, *et al.*, 1992). Lactic acid production is a product inhibited process with a poorly defined mechanism. The accepted mechanism involves the solubility of the non-dissociated form within the cytoplasm and the insolubility of the ionized acid form (Gatje, *et al.*, 1991 and McDonald, *et al.*, 1990). The lactic acid inhibition process is non-competitive at every pH value (Ohara, *et al.*, 1992). After production and excretion to the outside of the cell, lactic acid reduces the external pH. The bacterial cells maintain alkaline cytoplasmic pH by proton extrusion by the H⁺ATPase and the electrogenic

uptake of K^+ . The synthesis and activity of the ATPases are regulated by internal pH (Kobayashi, 1985 and Axelsson, 1993). In batch cultures, at the end of log phase when external pH is low and large amount of external lactic acid are present, lactobacilli can barely transport the lactic acid out of the cell. ATP synthesized during this time is not for cell growth but for creating PMF in order to maintain an alkaline cytoplasm. A non-dissociated form of lactic acid accumulates in the cytoplasm. It is reported that this form of the organic acid is toxic to bacterial cells (Gatje, 1991, Monot, *et al.*, 1984 and Gatje, *et al.*, 1991). Over the pH range of 4.1-5.0, non-dissociated lactic acid inhibited growth of *L. helveticus* (Goncalves, *et al.*, 1997). Lactic acid bacteria cannot control their acid-base balance during starvation under acidic conditions, because the H^+ efflux driven by H^+ -ATPase ceases due to nutrient and energy limitation. When the internal pH reaches a threshold, the metabolism is damaged and the cells die. *L. casei* subsp. *rhamnosus* was inhibited by its own product, L-(+) lactic acid, at a K_i of 120 mM on MRS media and 360 mM on molasses in batch culture. Molasses may have some ions to bind the non-dissociated form of lactic acid. Immobilized *L. casei* subsp. *rhamnosus* produced less lactate when lactic acid was added into molasses media, again indicating lactic acid end-product inhibition. To resolve the problem of product inhibition, lactic acid must be continuously removed from the fermentation. A continuous process is a promising method for solving this problem.

The amount of lactic acid produced was dependent on the substrate concentration up to a certain point. The higher the substrate, the more lactic acid produced. The highest efficiency of substrate conversion to lactic acid was found using 8 % Brix molasses, which has approximately 50 g/l of fermentable sugars (sucrose, glucose and fructose). For immobilized cultures, at a dilution rate of 0.2/hr, the most lactic acid was produced from 8% Brix molasses without nutrient supplementation. When molasses concentration increased to 10 and 13% Brix, the same amount of lactic acid as from 8% Brix molasses was produced. When molasses concentration was increased to 18% Brix, less lactic acid was produced, indicating that the osmolarity of molasses was too high. Similar results were found at higher dilution rates. Lactic

acid concentration dropped with increased dilution rates. The higher the dilution rate, the lower lactic acid concentration (this study, and Tejayadi and Cheryan, 1995).

When molasses was clarified prior to fermentation, lactic acid production decreased 53%. This may be due to either nutrient removal or destruction. Clarified molasses supplemented with 1% (w/v) inactivated invertase showed 100% increase in lactic acid production over clarified molasses. Inactivated invertase provides protein and other growth factors but not as well as yeast extract. The invertase tested is a commercial preparation made of crude Baker's yeast. In batch fermentation, *L. casei* subsp. *rhamnosus* grew better and produced more lactic acid when grown in invertase supplemented molasses than in yeast extract supplemented molasses. When 0.2% (w/v) yeast extract was added to molasses, the molar conversion was 1.87. When the same amount of invertase was added to molasses, the molar conversion was 1.99. The productivities were 0.5 and 1.2 for yeast extract and invertase supplementation, respectively. This suggests that *L. casei* subsp. *rhamnosus* can more readily utilize glucose and fructose than sucrose. The same preferences were reported for starch fermentations for lactic acid production by this organism. When starch was hydrolyzed into glucose prior to fermentation, there was higher productivity and yield (Tsai and Moon, 1998). The greatest efficiency of lactic acid production in batch fermentation was observed when the sucrose in molasses was inverted by using 0.2% (w/v) invertase. No residual fermentable sugars were detected. There is no evidence that *L. casei* subsp. *rhamnosus* produces invertase. Invertase functioned both to invert the sucrose and as a nutrient supplement. For immobilized cell culture, invertase treated molasses, did not show improved lactic acid production at a low dilution rates, 0.2 and 0.4/hr, but showed a 31% improvement at a high dilution rate, 1.1/hr. At the low dilution rates, 0.2 and 0.4/hr, the column was saturated when fed with non-supplemented molasses. Molasses did not need to be inverted or supplemented when lactic acid was produced continuously by immobilized culture at a low dilution rate.

Lactobacilli require complex nutrients for growth, particularly B vitamins. Yeast extract provides nitrogen, nucleotides and vitamin sources for microorganisms but is relatively expensive. Other nitrogen sources such as corn steep liquor and malt sprouts have been used due to their relatively low price but they are required in higher concentrations and have to be supplemented with small amounts of yeast extract to bring about desired yields and productivity (Goksungur, *et al.*, 1997, and Hujanen and Linko, 1996). Lactic acid production rates are high when the growth rates are low, regardless of nitrogen supplementation (Amrane and Prigent, 1998). For *L. casei* subsp. *rhannosus*, high concentrations of nitrogen reduced the length of the lag phase, and resulted in high cell mass. The amount of lactic acid produced was dependent on the amount of fermentable sugars. Nitrogen supplements allowed the bacteria to survive longer. For batch fermentations, with pH control, the amount of lactic acid produced from molasses, supplemented with 0.2, 0.4 or 0.8% yeast extract, was about the same, even though the fermentation times varied, resulting in differing productivity. Yeast extract shortened the lag phase, increased cell mass and productivity. However, the relationship between cell mass and lactic acid production was not linear. For immobilized cell fermentations, supplemented molasses enhanced the lactic acid production by 81% at a dilution rate of 1.1/hr, but had no effect at a lower dilution rate. At this flow rate, bacterial wash out is higher and the extra nutrients may help replenish numbers inside the column. Other nutrient supplements, corn steep liquor, ammonium phosphate (dibasic) and invertase were not as effective as yeast extract. Lactobacilli prefer organic nitrogen sources to inorganic ones (Hujanen and Linko, 1996). Phosphate has been reported to stabilize L-LDH of *S. bovis* during extraction and purification (Wolin, 1964), and is a competitive inhibitor of FDP binding of L-LDH (Jonas, *et al.*, 1972). Free phosphate strongly inhibits streptococcal pyruvate kinase of the glycolytic pathway (Collins and Thomas, 1974, Crow and Pritchard, 1976, and Abbe and Yamada, 1982). Ammonium phosphate (dibasic) did not improve lactic acid production.

The immobilized column was scaled up from 10 to 400 ml working volume to allow for pH control. Without pH control, 15 g/l lactic acid was produced at a dilution rate of 0.04/hr from

0.4% yeast extract supplemented molasses, 8% Brix, the same amount as the small bioreactor. When the reactor was pH controlled, 20 g/l of lactic acid was produced under the same conditions. This indicates that fermentation pH plays a major role in lactic acid production. When molasses concentration increased from 8 to 13% Brix, 31±4 g/l of lactic acid was produced from a pH controlled system at this dilution rate.

In the pilot reactor, lactic acid was produced mainly in the first segment (78% of lactic acid produced). The pH dropped rapidly across this segment to about 3.5 where *L. casei* subsp. *rharnosus* does not function efficiently. The pilot reactor also showed an accumulation of sugars and lactic acid at the bottom of the bioreactor indicating column stratification and poor flow patterns which influenced lactic acid yield. This will require redesign of the column system and proper engineering of a new pilot system. The pilot reactor was operated in a non-aseptic environment. Sodium azide, an inhibitor of the electron transport chain, was used to minimize aerobic contamination. However, it also inhibited *L. casei* subsp. *rharnosus* growth, at a K_i of 2.1 mM. In aerobes, sodium azide interacts with heme a_3 and blocks the flow of electrons from cytochrome oxidase to O_2 (Stryer, 1988). It is an inhibitor of myeloperoxidase and catalase (Hasui, *et al.*, 1991). Sodium azide is also a mutagen (Owais, *et al.*, 1978). It induces base substitution but not frameshift mutations with no chromosome breaks. *L. casei* subsp. *rharnosus* lacks cytochrome oxidase and catalase. Lactobacilli use a manganese-catalyzed scavenging of superoxide (Kandler and Weiss, 1986). Sodium azide can bind to Mn (II), which may inactivate the functioning of the L-(+) lactate dehydrogenase (Rosson, *et al.*, 1984).

Recovery of lactic acid from an immobilized fermentation system will be similar to current practice. Anionic resins are used in lactic acid purification (Vaccari, *et al.*, 1993 and Senthuran, *et al.*, 1997). Amberlite IRA-400 adsorbs pure lactic acid at a capacity of at least 29 mg lactic acid/g resin at pH 8.0. Bound acid (95%) was recovered. Amberlite IRA-400 also adsorbed lactic acid from spent broth from immobilized fermentation at a capacity of 34 mg/g resin. Recovery was 56% by elution with 2N HCl, but should improve with optimization.

Price of lactic acid is high compared to many other bulk chemicals. The expansion of the lactic acid market into polymer industries will require a lower price for lactic acid monomer. Lactic acid for biodegradable plastic requires a target manufacture cost lower than the current cost (\$ 1.10 to 2.2/kg). From 1985 to 1994, the price of 88% strength food grade lactic acid was \$ 2.53/kg on average (Kharas, *et al.*, 1994), and was about \$ 1.50 to \$ 1.85/kg in early of 1995 (Lepree, 1995). A significant portion of cost of lactic acid production from microbial fermentation is based on substrate cost, especially for nitrogen sources, approximately 35 to 38% of total cost (Tejayadi, *et al.*, 1995). Currently, yeast extract is the primary supplement. Lower price nitrogen sources, such as corn steep liquor, corn hydrolysates and soy hydrolysates, can be used but they cause downstream problems in purification process as they contain contaminating materials. A small amount of yeast extract is needed to enhance bacterial growth. The media cost (carbohydrates and nutrient supplements) for lactic acid production currently is approximately \$0.34/kg. In this study, for batch fermentations, L-(+) lactic acid was produced at a productivity of 1.2 g/l-hr to a concentration of 50 g/l lactic acid. The molasses was supplemented with invertase, which is cost additive. Calculated substrate cost of lactic acid production from batch fermentations was \$0.35/kg, the same as the current cost. Lactic acid from non-supplemented molasses at a productivity of 2.8 g/l-hr had a production cost for carbohydrate of \$0.24/kg lactic acid. The residual molasses contained mainly sucrose. This production cost can be further reduced if the residual molasses are resold. For immobilized cultures, lactic acid carbohydrate production cost can be reduced from \$0.24 to \$0.075/kg after molasses resale (Table 17). This low cost of production can only be obtained from immobilized, continuous fermentations, since batch fermentations utilize all the substrate and need nutrient supplementation.

L-(+) lactic acid can be commercially produced from blackstrap molasses by the selected strain of microorganism using cell immobilization technique in conjunction with continuous operation. The productivity of immobilized cell fermentation was higher than batch fermentation. The maximal productivity can be achieved with a proper designed plant. This will

Table 17. Comparison of financial analysis of carbohydrates and nutrient supplements for lactic acid production between current industrial batch fermentations and batch and immobilized cell fermentations from this study.

| Method | Cost (\$/kg lactic acid produced) | |
|----------------------------|-----------------------------------|-----------------|
| | No molasses resale | Molasses resale |
| Batch (current production) | 0.34 | 0.34 |
| Batch (this study) | 0.35 | 0.35 |
| Immobilized culture | 0.24 | 0.075 |

minimize capital and operating costs. Although the lactic acid concentrations from immobilized cell fermentations were lower than from batch fermentations, residual molasses from immobilized cell fermentation can be reused, which will minimize production costs. The residual molasses has some residual sugars, especially sucrose. This residual molasses can be reused in the application for animal feed or it can be redirected into the bioreactor after lactic acid purification. The life span of the immobilized culture can be many years, as long as the bioreactor is fed. Granular activated carbon is acceptable for a solid support although it can be replaced with a synthetic support with longer life span. Over time, feed flows may be obstructed, resulting in media channelling and lower productivity. Contamination by other organisms are normally observed. The plant has to be carefully designed to prevent these events. Some chemicals that inhibit contamination must be tested besides sodium azide. Lactobacilli may be mutated to tolerate lower pH or higher temperature in order to minimize contamination. Immobilized cells in a bioreactor are in stationary phase. Fewer cells are in the product effluent comparing to batch fermentations, which, in turn, lowers cost of product recovery and lowers effluent Biological Oxygen Demand (BOD). Blackstrap molasses did not require nutrient supplementation for lactic acid production by immobilized cell fermentation. This will make easier for product recovery, and result in lower cost of production. A plant for lactic acid production by continuous, immobilized cell fermentation can be designed based on the results obtained.

LITERATURE CITED

- Abbe, K. and T. Yamada. 1982. Purification and properties of pyruvate kinase from *Streptococcus mutans*. J. Bacteriol. 149(1): 299-305.
- Aiba, S., A.E. Humphrey and N.F. Millis. 1973. Kinetics. In: Biochemical Engineering, second edition. Aiba, S., A.E. Humphrey and N.F. Millis (eds). Academic Press, New York&London. Chapter 4, pp. 92-127.
- Amrane, A. and Prigent, Y. 1998. Lactic acid production rates during the different growth phases of *Lactobacillus helveticus* cultivated on whey supplemented with yeast extract. Biotechnology Letters. 20 (4): 379-383.
- Anders, R.J., J.G. Cervený and A.L. Milkowski. 1989. U.S. patent 4,888,191 to Oscar Mayer Foods Corp., USA.
- Anders, R.J., J.G. Cervený and A.L. Milkowski. 1991. U.S. patent 5017391 to Oscar Mayer Foods Corp., USA.
- Anderson, L.C., D.L. Wise and J.F. Howes. 1976. Contraception. 13: 375.
- Appleby, C.A. and R.K. Morton. 1959. Lactic dehydrogenase and cytochrome b2 of baker's yeast: purification and crystallization. Biochemical Journal. 71 (3):492-499.
- Atkinson, B. and F. Mavituna. 1983. Processes. In: Biochemical Engineering and Biotechnology Handbook. Atkinson, B. and F. Mavituna (eds). Nature Press, New York. Chapter 14, pp. 1044-1048.
- Axelsson, L.T. 1993. Lactic acid bacteria: Classification and physiology. In: Lactic Acid Bacteria. Salminen, S. and A. von Wright (eds). Marcel Dekker, Inc., New York. pp. 1-63.
- Bahner, B. 1994. Lactic acid suppliers: worst fear not realized. Chem. Marketing. Reporter. March 21, 1994, pp.14.
- Baker, H. and O. Frank. 1975. Analysis of riboflavin and its derivatives in biologic fluids and tissues. In: Riboflavin. Rivlin, R.S. (ed). Plenum Press, New York/ London. pp. 49-98.
- Bender, G.R. and R.E. Marquis. 1987. Membrane ATPases and acid tolerance of *Actinomyces viscosus* and *Lactobacillus casei*. Applied and Environmental Microbiology. 53 (9): 2124-2128.
- Benninga, H. 1990. A history of lactic acid making: A chapter in the history of biotechnology. Benninga, H. (ed). Kluwer Academic Publishers, Dordrecht, The Netherlands. pp.417-451.
- Bettenbrock, K., U. Siebers, P. Ehrenreich and C-L. Alpert. 1999. *Lactobacillus casei* 64H contains a phosphoenolpyruvate-dependent phosphotransferase system for uptake of galactose, as confirmed by analysis of ptsH and different gal mutants. J. Bacteriol. 181 (1): 225-230.
- Booth, I.R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiology Reviews. 49: 359-378.
- Buchta, K. 1983. Lactic acid. In: Biotechnology, first edition. Volume 3. Rehm, H.G. and Reed, G. (eds). Weinheim, Verlag Chemie. pp. 409-417.

- Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews*. 87: 113-130.
- Cerning, J., C.M.G.C. Renard, J.F. Thibault, C. Boullianne, M. Landon, M. Desmazeaud and L. Topisirovic. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Appl. Env. Microbiol.* 60(11): 3914-3919.
- Chang, T.M.S. 1976. Biodegradable microcapsules containing enzymes, hormones, vaccines and other biologicals. *J. Bioengineering*. 1: 25.
- Chassy, B.M., E.M. Gibson and A. Giuffrida. 1978. Evidence for plasmid associated lactose metabolism in *Lactobacillus casei* subsp. *casei*. *Current Microbiology*. 1: 141-144.
- Collins, L.B. and T.D. Thomas. 1974. Pyruvate kinase of *Streptococcus lactis*. *J. Bacteriol.* 120(1): 52-58.
- Cook, G.M. and J.B. Russel. 1994. The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis*. *Current Microbiology*. 28: 165-168.
- Crow, V.L. and G.G. Pritchard. 1976. Purification and properties of pyruvate kinase from *Streptococcus lactis*. *Biochim. Biophys. Acta*. 438: 90-101.
- Crow, V.L. and G.G. Pritchard. 1977. Fructose 1,6-diphosphate-activated L- lactate dehydrogenase from *Streptococcus lactis*; kinetic properties and factors affecting activation. *Journal of Bacteriology*. 131(1): 82-91.
- Damalin, L.H., G.A. Dykes and A. von Holy. 1995. Biodiversity of lactic acid bacteria from food-related ecosystem. *Microbios*. 83: 13-22.
- Danner, H, M.Neureiter, L. Madzingaidzo, M. Gartner and R. Braun. 1998. *Bacillus steareothermophilus* for thermophilic production of L-lactic acid. *Applied Biochemistry and Biotechnology*. 70-72: 895-903.
- Datta, R., S.P. Tsai, P. Bonsignore, S. Moon and J. Frank 1995. Technological and economic potential of poly (lactic acid) and lactic acid derivatives. *FEMS Microbiology Reviews*. 16: 221-231.
- Datta, R. and S.P. Tsai. 1997. Lactic acid production and potential uses: A technology and economics assessment. *ACS symposium series*. 666: 224-236.
- Demirci, A. and A.L. Pometto III. 1995. Repeated batch fermentation in biofilm reactors with plastic composite supports for lactic acid production. *Appl Microbiol Biotechnol*. 43: 585-589.
- Demirci, A. and A.L. Pometto III. 1992. Enhanced production of D(-) lactic acid by mutants of *Lactobacillus delbrueckii* ATCC 9649. *J. Ind. Microbiol*. 11: 23-28.
- Dong, X.Y., S. Bai and Y. Sun. 1996. Production of L(+) lactic acid with *Rhizopus oryzae* immobilized in polyurethane foam cubes. *Biotechnol Lett*. 18 (2): 225-228.
- Dreissen, A.J.M., B. Poolman, R. Kiewiet and W.N. Konings. 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cationic exchanger. *Proceeding National Academy of Sciences USA*. 84: 6093-6097.

- Du, J., N. Cao, C.S. Gong and G.T. Tsao. 1998. Production of L-lactic acid by *Rhizopus oryzae* in a bubble column fermenter. *Applied Biochemistry and Biotechnology*. 70-72: 323-329.
- Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350-356.
- Dunn, R.L., J.P. English, J.D. Strobel, D.R. Cowsar and T.R. Tice. 1988. Preparation and evaluation of lactide/glycolide copolymers for drug delivery. In: Migliaresi C (ed) *Polymers in Medicine III*. Elsevier, Amsterdam. pp. 149-159.
- Duxbury, D.D. 1993. Lactic acid; Versatile ingredient now made in the USA. *Food Processing*. May: 97-98.
- Dykes, G.A., I. Geornaras, and A. Von Holy. 1995. Advantages of sucrose-dependent extracellular polysaccharide production to lactic acid bacteria in sucrose-rich environment. *Lett. Appl. Microbiol.* 21: 327-329.
- Earnshaw, R.G. 1992. The antimicrobial action of lactic acid bacteria: Natural food preservation systems. In: *The Lactic Acid Bacteria*, vol.1, The lactic acid bacteria in health and disease. Wood, B.J.B. (ed). Elsevier Applied Science, London. pp.211-232.
- Eikmeier, H.R. and H.J. Rehm. 1987. Stability of calcium alginate during citric acid production of immobilized *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 26: 105-111.
- Enomoto, K., M. Ajioka and A. Yamaguchi. US patent 5,310,865 (to Misui Tuatsu).
- Fritze, D. and D. Claus. 1992. Spore-forming, lactic acid producing bacteria of the genera *Bacillus* and *Sporolactobacillus*. In: *The Lactic Acid Bacteria*, vol. 2 : The genera of lactic acid bacteria. Wood, B.J.B. and W.H. Holzapfel (eds). Blackie Academic & Professional, Glasglow. pp. 368-391.
- Fuller, R. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology*. 66: 365-378.
- Gamal, R.F. and N.F. Gamal. 1984. Utilization of agricultural residues for lactic acid production by a local isolate of *Lactobacillus* sp. *Annals of Agricultural Science, Ain Shams Univ.* 29: 145-152.
- Garvie, E.I. 1980. Bacterial lactate dehydrogenase. *Microbiological Reviews*. 44 (1): 106-139.
- Gatje, G., and G. Gottschalk. 1991. Limitation of growth and lactic acid production in batch and continuous cultures of *Lactobacillus helveticus*. *Appl. Microbiol. Biotechnol.* 34: 446-449.
- Gatje, G., V. Muller and G. Gottschalk. 1991. Lactic acid excretion via carrier mediated facilitated diffusion in *Lactobacillus helveticus*. *Appl. Microbiol. Biotechnol.* 34: 778-782.
- Gibson, T. and R.E Gordon. 1974. *Bacillus*. In: *Bergey's Manual of Determinative Bacteriology*, eighth edition. Buchanan, R.E. and N.E. Gibbons (eds). Williams and Wilkins, Baltimore. pp.529-550.
- Goksungur, Y. and U. Guvenc. 1997. Batch and continuous production of lactic acid from beet molasses by *Lactobacillus delbrueckii* IFO 3202. *J. Chem. Tech. Biotechnol.* 69: 399-404.
- Goncalves, L.M.D., A. Ramos, J.S. Almeida, A.M.R.B. Xavier and M.J.T. Carrondo. 1997. Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. *Appl. Microbiol. Biotechnol.* 48: 346-350.

Gruber, P.R., E.S. Hall, J.J. Kolstad, M.L. Iwen, R.D. Benson and R.L. Borchardt. 1992. US patent 5,142,023 (to Cargill).

Hamanci, H. and D.D. Ryu. 1994. Production of L(+) lactic acid using immobilized *Rhizopus oryzae*. Appl. Biochem. Biotechnol. 44: 125-133.

Hammes, W.P. and R.F. Vogel. 1995. The genus *Lactobacillus*. In: The Lactic Acid Bacteria vol 2: The genera of lactic acid bacteria. Wood, B.J.B. and W.H. Holzapfel (eds). Blackie Academic & Professional, Glasglow. Chapter 3, pp. 19-54.

Hang, Y.D. 1989. Direct fermentation of corn to L(+) lactic acid by *Rhizopus oryzae*. Biotech. Lett. 11 (4): 299-300.

Hasui, M., Y. Hirayabashi, K. Hattori and Y. Kobayashi. 1991. Increased phagocytic activity of polymorphonuclear leukocytes of chronic granulomatous disease as determined with flow cytometric assay. J. of Laboratory and Clinical Medicine. 117 (4): 291-298.

Ho, K-L. G., A.L. Pometto III and P.N. Hinz. 1997. Optimization of L(+) lactic acid production by ring and disc plastic composite supports through repeated-batch biofilm fermentation. Applied and Environmental Microbiology. 63 (7): 2533-2542.

Holland, H.J., B.J. Tighe and P.L. Gould. 1986. Polymers for Biodegradable Medical Devices. I. The potential of polyesters as controlled macromolecular release systems. J. Controlled Release. 4: 155-160.

Holten, C.H. 1971. Lactic acid properties and chemistry of lactic acid and derivatives. Verlag Chemie, Germany.

Holzapfel, W.H. and B.J.B. Wood. 1995. Lactic acid bacteria in contemporary perspective. In: The Lactic Acid Bacteria, volume 2: The genera of lactic acid bacteria. Blackie academic & Professional, Glasglow. Chapter 1, pp. 1-6. Wood, B.J.B. and Holzapfel, W.H. (eds).

Hongo, M., Y. Nomura and M. Iwahara. 1986. Novel method of lactic acid production by electro dialysis fermentation. Applied and Environmental Microbiology. 52 (2): 314-319.

Hujanen, M. and Y-Y. Linko. 1996. Effect of temperature and various nitrogen sources on L(+) lactic acid production by *Lactobacillus casei*. Applied Microbiology and Biotechnology. 45: 307-313.

Inskeep, G.C., G.G. Taylor and W.C. Breitzke. 1952. Lactic acid from corn sugar; a staff-industry collaborative report. Ind. Eng. Chem. 44 (9): 1955-1966.

ITC, International Trade Center, UNCTAD/GATT. 1972. The market for sucrose-based chemicals with specific reference to citric acid, sorbitol and sugar esters. Geneva.

Iwasaki, K-I. 1993. Rapid continuous lactic acid fermentation by immobilized lactic acid bacteria for soy sauce production. Process Biochemistry. 28: 39-45.

Iwasaki, K-I., M. Nakajima and H. Sasahara. 1992. Porous alumina beads for immobilization of lactic acid bacteria and its application for repeated-batch fermentation in soy sauce production. Journal of Fermentation and Bioengineering. 73 (5): 375-379.

Jeanet, R, J.L. Maubois and P. Boyaval. 1996. Semicontinuous production of lactic acid in a bioreactor coupled with nanofiltration membranes. Enzyme and Microbial Technology. 19: 614-619.

- Jin, L.Z., Y.W. Ho, M.A. Ali, N. Abdullah, K.B. Ong and S. Jalaludin. 1996. Adhesion of *Lactobacillus* isolates to intestinal epithelial cells of chicken. *Lett. Appl. Microbiol.* 22: 229-232.
- Jonas, H.A., R.F. Anders and G.R. Jago. 1972. Factors effecting the activity of the lactate dehydrogenase of *Streptococcus cremoris*. *J. Bacteriol.* 111:397-403.
- Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek.* 49 (3): 209-224.
- Kandler, O. 1984. Current taxonomy of lactobacilli. *Dev. Ind. Microbiol.* 25: 109-123.
- Kandler, O. and N. Weiss. 1986. Regular, nonsporing Gram-positive rods. In: *Bergey's Manual of Systemic Bacteriology*, vol.2. Krieg, N.R. and J.G. Holt (eds). Williams and Wilkins, Baltimore/ London. Section 14, pp. 1208-1234.
- Kashket, E.R. 1987. Biogenetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol. Rev.* 46: 233-244.
- Kharas, G.B., F. Sanchez-Riera and D.K. Severson. 1994. Polymers of lactic acid. In: *Plastics from Microbes*. Mobley, D.P. (ed). Carl Hanser Verlag, New York. pp.93-137.
- Kobayashi, H. 1985. A proton translocating ATPase regulates pH of the bacterial cytoplasm. *J. Biol.Chem.* 260: 72-76.
- Kolstad, J. 1996. *JAPS.* 62: 1079-1091.
- Konings, W.N., B. Poolman and A.J.M. Driessen. 1989. Bioenergetics and solute transport in lactococci. *Critical Reviews in Microbiology.* 16: 419-476.
- Lepree, J. 1995. Lactic acid prices rise as use tightens supply. *Chem. Marketing. Reporter.* March 13, 1995, pp. 14.
- Lerner, M. 1995. ADM is formidable biochem competitor. *Chem. Marketing. Reporter.* July 24, 1995, pp. 3.
- Li, S.M., H. Garreau and M. Vert. 1990. Structure-property relationships in the case of the biodegradation of massive aliphatic poly (alpha -hydroxy acids) in aqueous media. Part 1. Poly (DL-lactic acid). Part 2. Degradation of lactide-glycolide copolymers: PLA 37.5GA25 and PLA75GA25. *J. Mater. Sci. Med.* 1: 123-139.
- Lipinsky, E.S. and R.G. Sinclair. 1986. *Chem. Eng. Prog.* 82: 26.
- Lockwood, L.B. 1979. Production of organic acids by fermentations. In: *Microbial Biotechnology*. Peppler, H.J. and Perlman, D. (eds). second edition. Academic Press, Inc., New York. pp. 373-376.
- London, J. 1990. Uncommon pathways of metabolism among lactic acid bacteria. *FEMS Microbiology Reviews.* 87: 103-112.
- Lowe, C.E. 1954. US patent 2,668,162 (to Dupont).
- Lunt, J. 1998. Large scale production, properties and commercial applications of polylactic acid polymers. *Polymer Degradation and Stability.* 59: 145-152.
- McDonald, L.C., H.P. Fleming and H.M. Hassan. 1990. Acid tolerance of *Leuconostoc mesenteroids* and *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* 56: 2120-2124.

- Maloney, P.C. 1990. Microbes and membrane biology. *FEMS Microbiology Reviews*. 87: 91-102.
- Mason, N., C. Thies and T.J. Cicero. 1976. *J. Pharm. Sci.* 65: 847.
- Mattiasson, B. 1983. Immobilization methods. In: *Immobilized Cells and Organelles*. CRC Press, Boca Raton, Florida. Volume 1, chapter 2, pp. 3-25.
- Mayr, U., R. Hensel, M. Deparde, H.E Pauly, G. Pfeiderer and W.E Trommer. 1982. Structure-function relationship in the allosteric L-lactate dehydrogenase from *Lactobacillus casei* and *Lactobacillus curvatus*. *European Journal of Biochemistry*. 126: 549-558.
- Mayr, U., R. Hensel and O. Kandler. 1980. Factors affecting the quaternary structure of the allosteric L- lactate dehydrogenase from *Lactobacillus casei* and *Lactobacillus curvatus* as investigated by hybridization and ultracentrifugation. *European Journal of Biochemistry*. 110: 527-538.
- McCoy, M. 1998. Chemical markers try biotech paths. *Chemical and Engineering news*. 76 (25): 13-19.
- McGroarty, J.A. 1994. Cell surface appendages of lactobacilli. *FEMS Microbiology Letters*. 124: 405-410.
- McGroarty, J.A. 1993. Probiotic use of lactobacilli in the human female urogenital tract. *FEMS Immunol. Med. Microbiol.* 6: 251-264.
- Meade, G.P. and J.C.P. Chen. 1977. Composition of cane and juice. In: *Cane Sugar Handbook*. Wiley-Interscience Publication, Toronto. Chapter 2, pp. 15-29.
- Meade, G.P. and J.C.P. Chen. 1977. Outline process for manufacturing raw cane sugar. In: *Cane Sugar Handbook*. Wiley-Interscience Publication, Toronto. Chapter 4, pp. 43-47.
- Merck index. 1989. An Encyclopedia of Chemicals, Drugs and Biologicals, the eleventh edition. Merck & Co, Inc., Rahway, New Jersey. pp. 842.
- Miall, L.M. 1978. Organic acids. In: *Primary Products of Metabolism (Economic microbiology, vol. 2)*. A.H. Rose (ed). Academic Press, Inc., London. .pp. 95-98.
- Monedero, V., M.J. Gosalbes and G.P Martinez. 1997. Catabolite repression in *Lactobacillus casei* ATCC 393 is mediated by CcpA. *J. Bacteriol.* 179 (21): 6657-6664.
- Monot, F, J.M. Engasser and H. Petidemange. 1984. Influence of pH and undissociated butyric acid on the production of acetone and butynol in batch cultures of *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* 19: 422-426.
- Monteagudo, JM., J. Rincon, L. Rodriguez, J. Fuertes and A. Moya. 1993. Determination of the best nutrient medium for the production of L-lactic acid from beet molasses: a statistical approach. *Acta Biotechnology*. 13:103-110.
- Monteagudo, JM., J. Rincon, L. Rodriguez and J. Fuertes. 1997. Kinetics of lactic acid fermentation by *Lactobacillus delbrueckii* grown on beet molasses. *J. Chem. Tech. Biotechnol.* 68: 271-276.
- Monteagudo, JM., J. Rincon, L. Rodriguez and J. Fuertes. 1994. Optimization of the conditions of the fermentation of beet molasses to lactic acid by *Lactobacillus delbrueckii*. *Acta Biotechnol.* 14: 251-260.

- Morishita, T., Y. Deguchi, M. Yajima, T. Sakurai and T. Yura. 1981. Multiple nutritional requirements of lactobacilli: Genetic lesions affecting amino acid biosynthetic pathways. *Journal of Bacteriology*. 148 (1): 64-71.
- Muntyan, M.S., I.V. Mesyanzhinova, Y.M. Milgrom and V.P. Skulachev. 1990. the F1- type ATPase in anaerobic *Lactobacillus casei*. *Biochimica Biophysica Acta*. 1016: 371-377.
- Nagodawithana, T. 1998. Production of flavours. In: *Nutritional Requirements of Commercially Important Microorganisms*. Esteekey associates, Milwaukee. Chapter 12, pp. 302-303.
- Nakano, K., H. Kataoka and M. Matsumura. 1996. High density culture of *Propionibacterium freudenreichii* coupled with propionic acid removal system with activated charcoal. *J. Fermentation and Bioengineering*. 81 (1): 37-41.
- Neidhardt, F.C., J.L. Ingraham and M. Schaechter. 1990. Biosynthesis and fueling. In: *Physiology of the Bacterial Cell: A molecular approach*. Sinauer Associates, Sunderland, Massachusetts. Chapter 5, pp. 133-173.
- Ogawa, Y. 1997. Injectable microcapsules prepared with biodegradable poly(alpha -hydroxy) acids for prolonged release of drugs. *J. Biomater. Sci. polymer edition*. 8 (5): 391-409.
- Ogawa, Y. 1992. *Eur. J. Hospital Pharm.* 2: 120.
- Ohara, H., K. Hiyama and T. Yoshida. 1992. Kinetic study on pH dependence of growth and death of *Streptococcus faecalis*. *Appl. Microbiol. Biotechnol.* 38: 403-407.
- Ohara, H., K. Hiyama and T. Yoshida. 1992. Non-competitive product inhibition in lactic acid fermentation from glucose. *Appl. Microbiol. Biotechnol.* 36: 773-776.
- Ohara, H., K. Hiyama and T. Yoshida. 1993. Lactic acid production by a filter-bed-type reactor. *J. Fermentation and Bioengineering*. 76 (1-2): 73-75.
- Olbrich, H. 1970. *Geschichte der Melasse*. Berlin, Bartens. pp.688.
- Owais, W.M., M.A. Zarowitz, R.A. Gunovich, A.L. Hodgdon, A. Kleinhofs and R.A. Nilan. 1978. A mutagenic in vivo metabolite of sodium azide. *Mutation Research*. 53: 355-358.
- Patureau, J.M. 1969. *Byproducts of the Cane Sugar Industry*. Amsterdam, Elsevier.
- Pelletier, C., C. Bouley, C. Cayuela, S. Boutier, P. Bourlioux and M-N Bellon-Fontaine. 1997. Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus* strains. *Applied and Environmental Microbiology*. 63 (5): 1725-1731.
- Pennings, A.J. 1989. Synthesis, properties and biomedical applications of poly(L-lactide). Paper presented at Symposium on "Biodegradable Carbohydrate-based Polymers". Amsterdam. November 13.
- Peppler, H.J. 1967. Yeast technology. In: *Microbial Technology*. Peppler, H.J. (eds). Reinhold, New York. pp. 145-171.
- Piskin, E., A. Tuncel, A. Denizli, E.B. Denkbaz, H. Ayhan, H. Cicek and K.T. Xu. 1994. Nondegradable and biodegradable polymeric particles. Preparation and some selected biomedical applications. In: *Diagnostic Biosensor Polymers*. Usmani, A.M. and N. Akmal (eds). ACS Symposium Series 556. American Chemical Society, Washington, DC. Chapter 18, pp. 222-237.

Poolman, B., K.J. Hellingwerf and W.N. Konings. 1987. Regulation of the glutamate-glutamine transport system by intracellular pH by *Streptococcus lactis*. J. Bacteriol. 169: 2272-2276.

Purac, Inc. 1989. Lactic acid and lactates. Product bulletin, Purac, Inc., Arlington Heights, Ill.

Rogosa, M., R.F Wisemann, J.S. Mitchell and M. Disraely. 1953. Species differentiation of oral lactobacilli from man including description of *Lactobacillus salivarius* nov, spec. and *Lactobacillus cellobiosus* nov, spec. J. Bacteriol. 65: 681-699.

Rosson, R.A., B.M. Tebo, and K. H. Nealson. 1984. Use of poisons in determination of microbial manganese binding rates in seawater. Appl. Env. Microbiol. 47 (4): 740-745.

Roukas, T. and P. Kotzekidou. 1998. Lactic acid production from deproteinized whey by mixed cultures of free and coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells using fedbatch culture. Enzyme and Microbial Technology. 22: 199-204.

Roy, D., J. Goulet and A. LeDuy. 1987. Continuous production of lactic acid from whey permeate by free and calcium alginate entrapped *Lactobacillus helveticus*. J. Dairy Sci. 70: 506-513.

Russel, J.B. and T. Hino. 1985. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that leads to rumen acidosis. J. Dairy Sci. 68: 1712-1721.

Russel, J.B., D.R. Bond and G.M. Cook. 1996. The fructose diphosphate/phosphate regulation of carbohydrate metabolism in low G+C Gram positive anaerobes. 14th Forum in Microbiology: Regulation of carbon metabolism in bacteria. pp. 528-535.

Ruter, P. 1975. Molasses utilization. Food and Agricultural Organization of the United Nations, Rome.

Schleifer, K.H. and W. Ludwig. 1995. Phylogenic relationships of lactic acid bacteria. In: The Lactic Acid Bacteria, vol.2: The genera of lactic acid bacteria. Wood, B.J.B. and W.H. Holzapfel (eds). Blackie Academic& Professional, Glasglow. Chapter 2, pp. 7-18.

Senthuran, A., V. Senthuran, B. Mattiasson and R. Kual. 1997. Lactic acid fermentation in a recycle batch reactor using immobilized *Lactobacillus casei*. Biotechnol. Bioeng. 55(6): 841-853.

Severson, D.K. 1998. Lactic acid fermentations. In: Nutritional Requirements of Commercially Important Microorganisms. Esteekay associates, Milwaukee. Chapter 11, pp. 258-297.

Singh, J. and B. Ranganathan. 1977. Acid production and proteolytic activity in milk by gamma-irradiation induced mutants of lactobacilli. J. Food Protection. 40 (9): 600-602.

Soccol, C.R., B. Marin, M. Raimbault and J.M. Lebeault. 1994. Potential of solid state fermentation for production of L- (+) lactic acid by *Rhizopus oryzae*. Appl. Microbiol. Biotechnol. 41: 286-290.

Strittermatter, C.F. 1959. Electron transport to oxygen in lactobacilli. J. Biol. Chem. 234: 2789-2793.

Strittermatter, C.F. 1959. Flavin-linked oxidative enzymes of *Lactobacillus casei*. J. Biol. Chem. 234: 2794-2800.

Stryer, L. 1988. Oxidative phosphorylation. In: Biochemistry. Chapter 17, pp. 412-413.

- Sumner, J.B. 1921. J. Biol. Chem. 47, 5.
- Taylor, K.A.C.C. 1996. A simple colorimetric assay for muramic acid and lactic acid. *Applied Biochemistry and Biotechnology*. 56:49-58.
- Tejayadi, S and M. Cheryan. 1995. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. *Applied Microbiology and Biotechnology*. 43: 242-248.
- Thayer, A. 1997. Polylactic acid is basis of Dow, Cargill venture. *Chemical and Engineering News*. 75 (49): 14-16.
- Toennies, G. and D.L. Gallant. 1949. The relationship between photometric turbidity and bacterial concentration (bacteriometric studies IV). *Growth* 13:7-20.
- Torres, A., S.M. Li, S. Roussos and M. Vert. 1996. Screening of microorganisms for biodegradation of poly (lactic acid) and lactic acid-containing polymers. *Appl. Environ. Microbiol.* 62 (7): 2393-2397.
- Tsai, S.P. and S.H. Moon. 1998. An integrated bioconversion process for production of L-lactic acid from starchy potato feedstocks. *Appl. Biochem. Biotech.* 70-72: 417-428.
- Tsai, S.P., R.D. Coleman, S.H. Moon, K.A. Schneider and C. Sanville Millard. 1993. Strain screening and development for industrial lactic acid fermentation. *Appl. Biochem. Biotechnol.* 39/40: 323-335.
- Tseng, C.P. and T.J. Montville. 1993. Metabolic regulation of end product distribution in lactobacilli: causes and consequences. *Biotechnology Progress*. 9: 113-121.
- Tuli, A., R.P. Sethi, P.K. Khanna and S.S. Marwaha. 1985. Lactic acid production from whey permeate by immobilized *Lactobacillus casei*. *Enzyme Microb. Technol.* 7: 164-168.
- Vaccari, G., R. Gonzales-Vara, A.L. Campi, E. Dosi and P. Brigidi. 1993. Fermentative production of L-lactic acid by *Lactobacillus casei* DSM 20011 and product recovery using ion exchange resins. *Appl. Microbiol. Biotechnol.* 40: 23-27.
- Van Ness, J.H. 1981. Hydroxy carboxylic acids. In: Kirk Othmer Encyclopedia of Chemical Technology, third edition, J. Wiley & Sons, New York. 13: 80-103.
- Vert, M., S.M. Li and H. Garreau. 1991. More about the degradation of LA-GA derived matrices in aqueous media. *J. Controlled Release*. 16: 15-26.
- Vert, M., A. Torres, S.M. Li, S. Roussos and H. Garreau. 1994. The complexity of the biodegradation of poly (alpha -hydroxyacid)-type aliphatic polyesters. In: *Biodegradable Plastics and Polymers*. Y. Doi and K. Fukuda (eds). Elsevier, Amsterdam. pp. 11-23.
- Vickroy, T.B. 1985. Lactic acid. In: *Comprehensive Biotechnology*, vol. 3. M. MooYoung (ed). Pergamon Press, Oxford. pp. 761-775.
- Wang, H., M. Seki and S. Furusaki. 1995. Mathematical model for analysis of mass transfer for immobilized cells in lactic acid fermentation. *Biotechnology Progress*. 11: 558-564.
- Wolin, M.J. 1964. Fructose 1,6-diphosphate requirement of streptococcal lactate dehydrogenase. *Science*. 146: 775-777.

- Xavier, S and B.K. Lonsane. 1994. Sugar-cane pressmud as a novel and inexpensive substrate for production of lactic acid in a solid-state fermentation system. *Appl. Microbiol. Biotechnol.* 41: 291-295.
- Yamada, T. and J. Carlsson. 1975. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. *J. Bacteriol.* 124 (1): 55-61.
- Yang, C.W., Z. Lu and G.T. Tsao. 1995. Lactic acid production by pellet-form *Rhizopus oryzae* in a submerged system. *Appl. Biochem. Biotechnol.* 51/52: 57-71.
- Yang, M, M. Yoshimura, M. Okada, S. Nagai and H. Sunahara. 1992. Anaerobic digestion using a fixed-bed reactor with bacteria immobilized by polyacrylate on a saddle-shaped slag support. *J. Fermentation and Bioengineering.* 73 (4): 303-307.
- Yoo, I-K., H.N. Chang, E.G. Lee, Y.K. Chang and S-H Moon. 1997. By-product formation in cell-recycled continuous culture of *Lactobacillus casei*. *Biotechnology Letters.* 19 (3): 237-240.
- Yoo, I-K., H.N. Chang, E.G. Lee, Y.K. Chang and S-H Moon. 1997. Effect of B vitamin supplementation on lactic acid production by *Lactobacillus casei*. *J. Ferment. Bioeng.* 84 (2): 172-175.
- Yu, R. and Y.D. Hang. 1989. Kinetics of direct fermentation of agricultural commodities to L(+) lactic acid by *Rhizopus oryzae*. *Biotech. Lett.* 11 (8): 597-600.
- Yu, W.P., J.P. Wong and T.M.S. Chang. 1998. Preparation of polylactic acid microcapsules containing Ciprofloxacin. *J. Microencapsulation.* 15 (4): 515-523.
- Zakaria, Z., G.M. Hall and G. Shama. 1998. Lactic acid fermentation of scampi waste in a rotating horizontal bioreactor for chitin recovery. *Process Biochemistry.* 33 (1): 1-6.
- Zayed, G. and J. Winter. 1995. batch and continuous production of lactic acid from salt whey using free and immobilized cultures of lactobacilli. *Appl. Microbiol. Biotechnol.* 44: 382-366.
- Zhu, Y., J.P. Smits, W. Knol and J. Bol. 1994. A novel solid state fermentation system using polyurethane foam as inert carrier. *Biotechnology Letters.* 16 (6): 643-648.

VITA

Narumol Thongwai was born on February 4, 1966, in Udonthani, Thailand. After graduating from the Udonpittayanukul High School in 1984, she attended the ChiangMai University where she obtained her bachelor of science degree in Medical Technology in 1988 with a silver medal.

From 1988 to 1993, Narumol Thongwai had been working at Siriraj Hospital, Mahidol University, Bangkok, as a research assistant in the Department of Immunology. In 1994, she earned a 5-year scholarship from the Ministry of Science and Technology to continue her education to earn a doctoral degree on the requirement of the ChiangMai University, department of biology programming in microbiology. She began her master's program in the Department of Microbiology at Louisiana State University in Baton Rouge in 1994 and switched to the doctoral program in 1995. She will receive the degree of Doctor of Philosophy in August 1999.

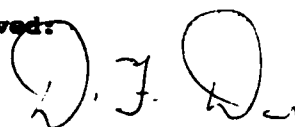
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Narumol Thongwai

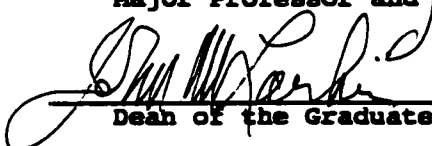
Major Field: Microbiology

Title of Dissertation: Production of L-(+) Lactic Acid From Blackstrap Molasses by Lactobacillus casei Subspecies Rhamnosus ATCC 11443

Approved:

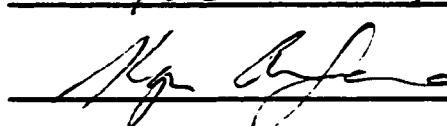

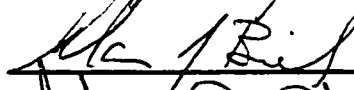
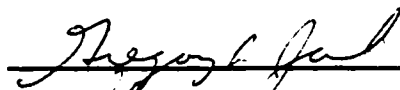


Major Professor and Chairman



Dean of the Graduate School

EXAMINING COMMITTEE:



Date of Examination:

13 May 1999